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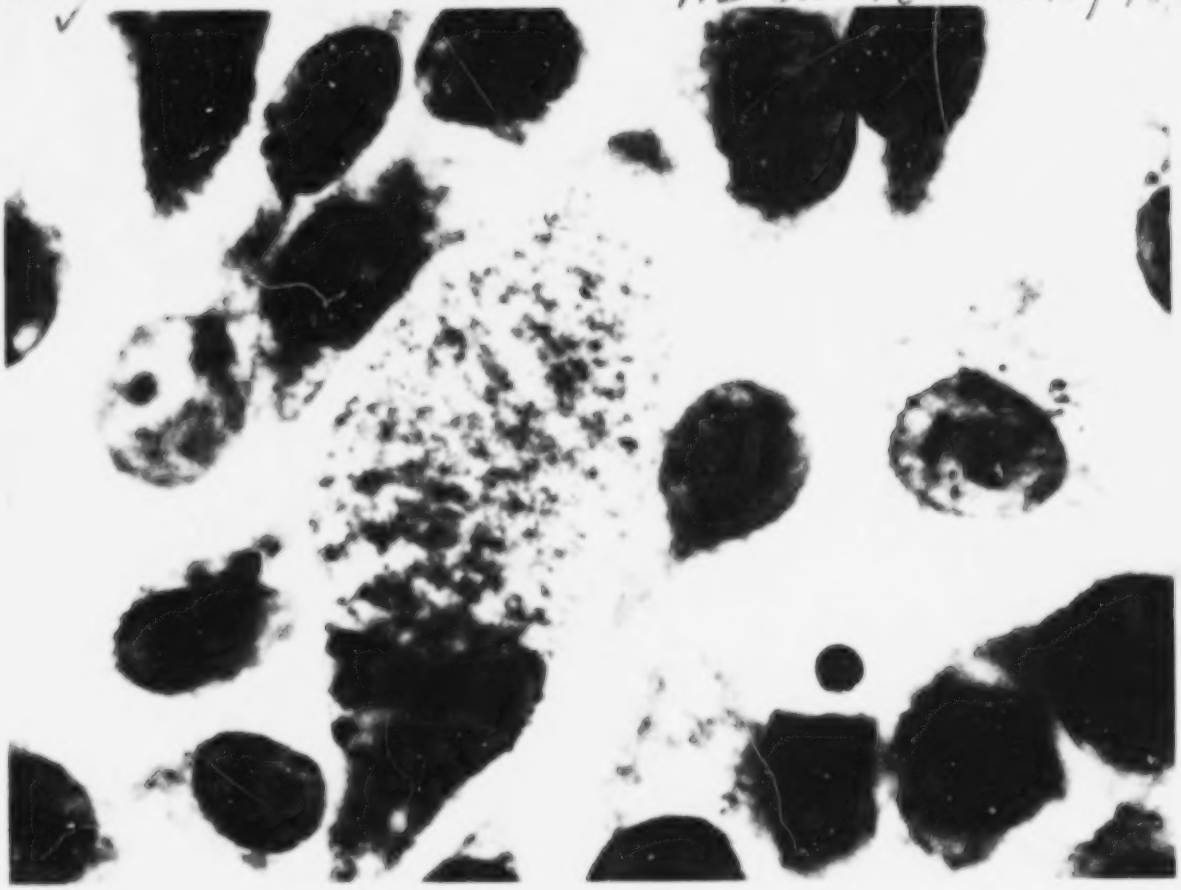

Laboratory Diagnosis of *Chlamydia trachomatis* Infections

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U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
PUBLIC HEALTH SERVICE
CENTERS FOR DISEASE CONTROL

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LABORATORY DIAGNOSIS OF *CHLAMYDIA TRACHOMATIS* INFECTIONS

by
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OCTOBER 1981

U.S. DEPARTMENT OF HEALTH AND HUMAN SERVICES
Public Health Service
Centers for Disease Control
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INTRODUCTION

In recent years, the incidence of sexually transmitted diseases has increased at an alarming rate and has become a major health problem throughout the world. There is a desperate need to bring these diseases under control. Not only do the sexually transmitted diseases cause untold suffering in the adult population but they also threaten the fetus and newborn as well. Control of these diseases, however, is a difficult task due to the many factors, both social and medical, that are involved.

In examining some of the factors, it is found that changes in the patterns of sexual activity and in the selection of contraceptive methods have led to the great increase of the sexually transmitted diseases. In addition to the enormous number of symptomatic cases that are diagnosed and treated, there is an unknown number of asymptomatic or undiagnosed cases that persist as reservoirs, perpetuating the infections. Besides the organisms of gonorrhea and syphilis, there are numerous infectious agents that are sexually transmitted – other bacteria, viruses, fungi, protozoa, mycoplasmas, and Chlamydia. Various combinations of these organisms can coexist and be transmitted simultaneously. Therefore, multiple infections occur which must be recognized and dealt with accordingly. Most of the infectious agents respond to appropriate antibiotic therapy, although relapses can occur. Antibiotic-resistant strains of the organisms can develop; thus, routine drug therapy is ineffective. Many of the infections may persist in a dormant or latent state, becoming active months or years following the initial infection. Reinfection is not uncommon, particularly within certain populations. At present, the diseases cannot be prevented through immunization since effective vaccines are not available.

To effectively solve the many problems associated with control of sexually transmitted diseases, an interrelationship of the various disciplines within the health science field is required. This manual deals with one aspect of the problem – the laboratory diagnosis of Chlamydial infections. In many areas of world, Chlamydial infections have been recognized as one of the most common of the sexually transmitted diseases.

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PROPERTIES AND CHARACTERISTICS OF CHLAMYDIAE

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Introduction

The Chlamydiae are a group of parasitic organisms which infect vertebrates and may occasionally be associated with arthropods. They may elicit a variety of well-defined disease patterns, but exist more commonly in the natural host as subclinical infections. In the past, the Chlamydiae were thought to be viruses because, like viruses, they will not grow on artificial media. They differ from viruses, however, in that Chlamydiae contain both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), possess ribosomes, multiply by binary fission, are susceptible to certain antibiotics, and have a cell wall similar to bacteria. The Chlamydiae are nonmotile, gram-negative organisms that have a similar morphology, share a common group antigen, and possess some metabolically active enzymes. They go through a unique developmental cycle within the cytoplasm of their host's cells. These obligate intracellular parasites are now regarded as bacteria that lack some important mechanism for producing metabolic energy.

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Morphology

Chlamydiae have two morphological forms – the elementary body (EB) and the reticulate (RB) or initial body. The EB is a uniformly spherical particle about 0.3 micrometers (μm) in diameter (Figure 1) that has an electron-dense center and is enclosed by a limiting cell wall. The EB is infectious and can survive outside the host cell. Within the host cell, the EB undergoes morphological changes. During the developmental cycle, the EB reorganizes into large (up to 1 μm) structures – the reticulate bodies. The RB is less dense than the EB and has a highly permeable and extremely fragile cell envelope. It exists only intracellularly and is noninfectious.

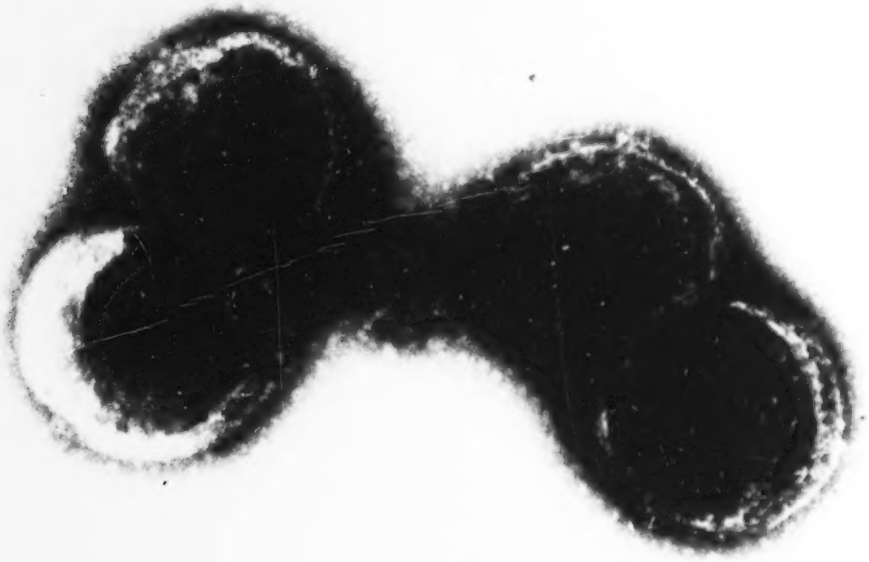

Figure 1. Electron Micrograph, Chlamydia Elementary Bodies (Courtesy of Dr. Vester Lewis)

Chemical Composition

The EB contains the complete circular genome of the Chlamydia. This DNA genome has a molecular weight of 660 x 10^6 daltons, which is larger than that of viruses (four times larger than Vaccinia) or mycoplasmas but is only one-fourth the weight of *Escherichia coli*. RNA is present in amounts equal to that of the DNA and exists as ribosomal RNA, ribosomal subunits, and transfer RNA. The EB

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also contains DNA-dependent RNA polymerase molecules necessary for the transcription of RNA from the DNA template. Protein substructures, such as certain amino acids, and large amounts of lipids, mostly phospholipids, are also constituents of the EB.

Effect of Physical Conditions and Chemical Agents

Temperature:

Chlamydiae are sensitive to heat. Infectivity is completely lost within 10 minutes at 60° C and is drastically reduced within 48 hours at 37° C. At 4° C, the Chlamydiae survive satisfactorily for about 24 hours. In freezing, an initial loss of infectivity occurs but thereafter the infectivity will remain stable at temperatures of –65° C or lower. Lyophilization greatly reduces the infectivity but the organisms that survive will remain infectious over a long period.

Stabilizers:

The stability of Chlamydiae suspensions is enhanced by adding proteins, lipids, or certain ions to the diluent. Several diluents that have shown to be satisfactory are (1) 10% skim milk (SM); (2) sucrose in a sodium phosphate buffer (SP); and (3) sucrose in potassium phosphate with glutamate (SPG).

Chemicals:

Chlamydiae are readily inactivated by (1) ether – within 30 minutes at room temperature; (2) formalin – 0.1% within 24 hours; and (3) phenol – 0.5% within 24 hours.

Antibiotics:

Certain antibiotics inhibit Chlamydiae replication. The penicillins and cycloserine, which act on the cell wall, may cause defective forms of the organisms to develop. These antibiotics interfere with the laboratory propagation of Chlamydiae, but are not effective in treating clinical infections. The tetracyclines, erythromycin, and chloramphenicol, which inhibit protein synthesis, not only inhibit chlamydial growth in the laboratory but are also effective in the treatment of clinical disease. Sulfonamides will inhibit members of the *Chlamydia trachomatis* group and have been used for treatment of disease. Other antibiotics such as rifampicin have also been found to be inhibitory, but as yet have not been widely adopted for routine laboratory or clinical use.

Streptomycin, neomycin, gentamicin, and the polymyxins have very little inhibitory effect and may be used in the laboratory for recovery and growth of the organism.

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Biological Characteristics

The Chlamydiae are prokaryotic obligate parasites of eukaryotic cells. Although the Chlamydiae contain certain enzymatic systems and can synthesize protein, they lack certain other systems necessary for extracellular development. The host-parasite relationship has yet to be fully understood, but it is known that the Chlamydiae cannot generate their own energy and must depend on energy supplied by the host cell. The host cell must have active mitochondria for Chlamydiae development to take place.

Within the host cell, the Chlamydiae go through a unique developmental cycle. An identifying characteristic of the Chlamydiae is the formation of an inclusion body in the cytoplasm of the host cell. The developmental cycle takes place inside the inclusion body. In appropriately stained preparations, the inclusions and the various stages of the developmental cycle can be seen. It was the observation of these inclusion bodies by Halberstaedter and Von Prowazek (1907) in Giemsa-stained, conjunctival scrapings from trachoma infections that formed the groundwork of chlamydial studies. They observed small reddish particles that they thought to be the agents of trachoma. These particles appeared to be contained in a blue matrix or mantle; thus they called them "chlamydozoa" or "mantle bodies." Today, we still use the observation of the inclusion bodies as a means of detecting and identifying Chlamydia-infected cells.

Developmental Cycle

The developmental cycle of all the Chlamydiae is similar. There are, however, certain differences between the species, most notably the production of glycogen by *C. trachomatis*.

Early information on the developmental cycle was supplied by microscopic examination of infected cells which had been stained. A useful stain for study of the developmental cycle is acridine orange, which differentiates cellular RNA and DNA. In recent years, with the development of more sophisticated techniques, new information at the molecular level has been acquired.

The Chlamydiae developmental cycle occurs in the cytoplasm of the host cell, and is characterized by formation of an intracytoplasmic inclusion body, inside of which the Chlamydiae develop. This membrane-bound inclusion may contain all the different forms of the developing Chlamydia. The cycle, from the entrance of the EB

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into the host cell, to the production of more mature infectious elementary bodies, takes about 48 hours.

The various stages of the developmental cycle have been observed not only in cells from in vitro cultures, but also in cells from conjunctival and cervical scrapings from human infections. The developmental cycle is described in Figure 2 on the following page.

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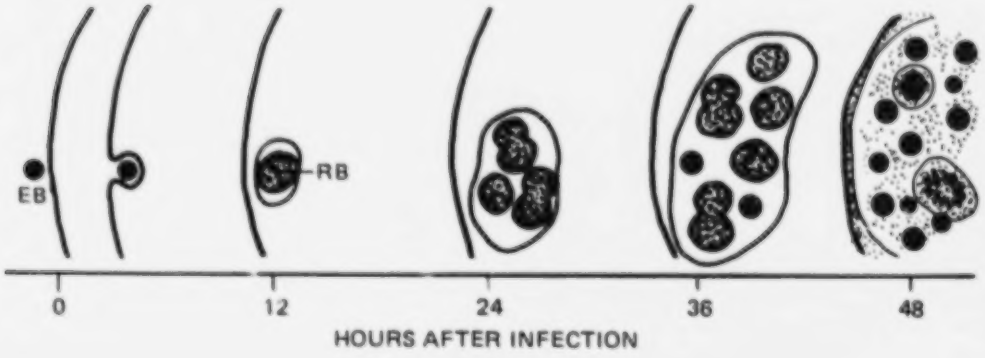
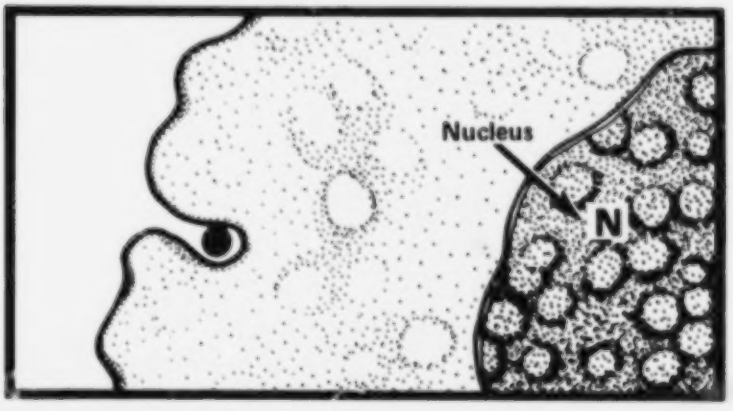
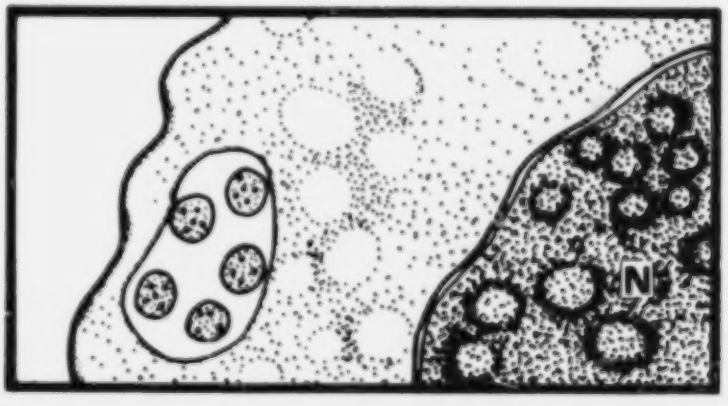
Figure 2. Events in the Development Cycle





TIME: 0-6 hours FORM (size): EB (0.3 μm)

Attachment and Penetration: The infectious EB attaches to the cell membrane and is taken into the cell by phagocytosis. The EB is enclosed by a vacuole made from the host cell membrane. As the EB reorganizes into reticulate bodies (RB), it loses its rigid shape and infectivity is lost. DNA and RNA are present in about equal amounts.



TIME: 6-24 hours FORM (size): RB (1.0 μm)

Reticulate Body Development: The RBs increase in number and size. The RBs are noninfectious, thus no infectivity can be detected during this period. RNA production begins and increases to an amount about three times that of the DNA. If infected cells are stained with

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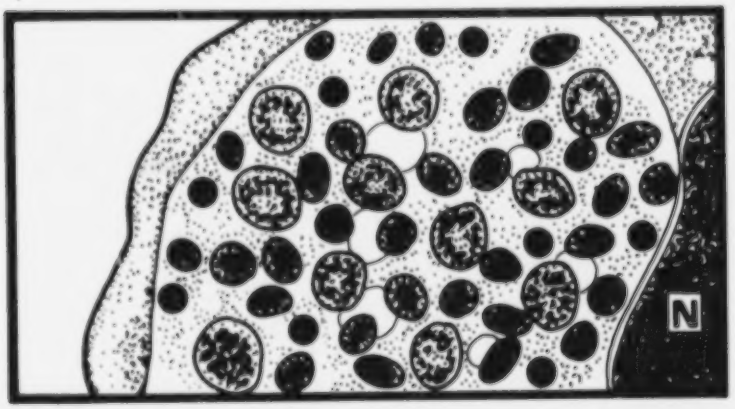
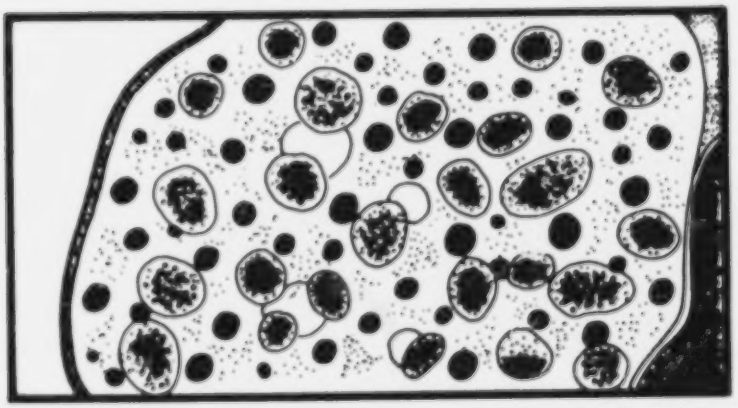
the fluorochrome acridine orange, the inclusion will stain red (indicating RNA); thus, this stage is known as the "red ball" stage. In other stained preparations such as Giemsa or in electron micrographs (EM), the heterogenous RBs can be observed around the periphery of the inclusion. Towards the end of this period, protein, DNA, and glycogen synthesis begins. The RBs begin undergoing binary fission.



TIME: 24-48 hours FORM: RB, EB

Elementary Body Maturation: Binary fission continues with the formation of immature elementary bodies, (0.35-0.5 μm). These bodies condense to become mature, infectious EBs. As RNA production decreases, DNA production increases. With DNA production, infectivity is again detectable. Glycogen production increases and peaks during this period.



TIME: 48-72 hours FORM: EB

Completion: Infectivity reaches its maximum. Glycogen is degraded and will eventually become undetectable. The inclusion becomes filled with mature EBs which may be liberated with destruction of the host cell.

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Classification and Terminology

The organisms that are today known as Chlamydia have in the past had a variety of names and classifications. In the early 1950's, the agents of trachoma and inclusion conjunctivitis were accepted as members in the psittacosis – lymphogranuloma venereum (LGV) group of viruses. This group became known as the PLT group of atypical viruses. This nomenclature persisted until the mid-1960's when the name "Bedsoniae" came into use. About this same time, microbiologists recognized that these organisms were not viruses, but bacteria, and assigned them to a separate genus. Within the genus, subgroups were recognized and designated Subgroup A and B. These subgroups have now been designated as the two species *Chlamydia trachomatis* and *Chlamydia psittaci*. The present classification became established in the early 1970's when Chlamydiales was recognized as a distinct order.

ORDER:	Chlamydiales	
FAMILY:	Chlamydiaceae	
GENUS:	*Chlamydia*	
SPECIES:	*C. psittaci*	*C. trachomatis*

Species Characteristics

C. psittaci (Subgroup B): Primarily animal pathogens

Psittacosis – Ornithosis
Meningopneumonitis
Feline pneumonitis

C. trachomatis (Subgroup A): Primarily human pathogens

Trachoma
Inclusion Conjunctivitis
Lymphogranuloma Venereum (LGV)

Assignment of the Chlamydia to a particular species is based on the nature of the intracytoplasmic inclusion bodies and susceptibility to sulfonamides and cycloserine, as indicated in Figure 3. Species-specific antigens and DNA homology further characterize the two species.

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Figure 3. Characteristics of the Chlamydia Species

FACTORS	*C. psittaci*	*C. trachomatis*
Nature of the Inclusion Bodies	Diffuse	Compact
	Non-Rigid Wall	Rigid Wall, Compressing Nucleus
	Glycogen Absent	Glycogen Present
	Iodine Stain Negative	Iodine Stain Positive
Sensitivity to Sulfonamides and Cycloserine	Not Sensitive	Sensitive

The sexually transmitted Chlamydia are members of the species *C. trachomatis*, which includes LGV and the traditional "TRIC" agents (Trachoma–Inclusion Conjunctivitis). Although the taxonomic nomenclature is straightforward, the everyday terminology used by workers in the field of sexually transmitted diseases is sometimes confusing. The term "TRIC" has been useful in dealing with diseases of the eye, but with genital infections, it becomes confused with Trichomonas–commonly referred to as "Tric" by those who deal with venereal infections. The term "Chlamydia," although commonly used, is not definitive since all members of the genus could be included. It has been suggested that the term "trachoma organisms" be used. Here again, delineation is a problem since trachoma is a disease of the eye. For the present, the problem of terminology must be recognized, and the terms used should be carefully selected.

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Antigens and Immunotypes

The Chlamydia organisms have multiple antigens. The composition and interrelationship of these antigens are rather complex and have yet to be fully understood. The antigens may be broadly grouped, however, into two main categories: group antigens, which are shared by all members of the Chlamydia genus, and specific antigens, which are more intraspecies related.

Group Antigens

Group antigens, common to all members of the Chlamydia genus, are heat stable and composed of lipoproteins and carbohydrates in association with the cell wall. They are reactive in the complement fixation, hemagglutination, and enzyme-linked immunosorbent assay (ELISA) tests.

Specific Antigens

Species-Specific Antigens:

Antigens which are specific for the two Chlamydia species are associated with the cell wall and are heat labile. Although they may be uncovered by removal of the group antigen components, the recovery is difficult since the specific antigens are easily destroyed by purification treatments.

Type-Specific Antigens:

Closely associated with the infectious organisms (EB) is a toxic material which is lethal for mice. This "toxin" differs antigenically with different chlamydial types and can be neutralized by specific antibody. Through the use of the mouse toxicity prevention test (MTPT), a toxin neutralization test, and the micro-immunofluorescence test (micro-IF), which detects surface antigens, 15 immunotypes of *C. trachomatis* have been recognized. There are 12 types, designated alphabetically A through K plus B_a, that have been identified for the "TRIC" agents and 3 types for LGV. The antigenic relationships among the types are quite complex. Certain types are so closely related that differentiation is difficult without extensive cross-testing. LGV types 1 and 2 are broadly cross-reactive. Figure 4 lists the immunotypes and indicates those that are highly related.

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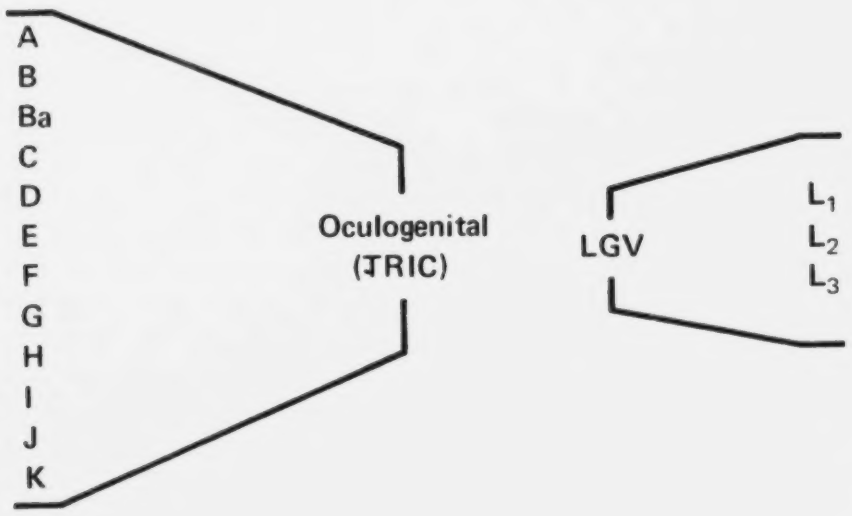
Strain Nomenclature

In the early years, there was no organization in the naming of isolates. Each laboratory had its own system of nomenclature. In the early 1960's, however, an orderly system was designed which has since been adopted by many laboratories. For full identification, a strain may be designated as follows:

Isolate Information	Example
Agent	TRIC
Immunotype	D
Geographic Location	USA, Washington
Laboratory and Number	University of Washington – 5
Origin of Clinical Specimen	Cervix

With abbreviations, the isolate would be identified: TRIC/D/USA.Wash./UW-5/Cx. For simplicity, a shorter version is commonly used: D/UW-5/Cx.

Figure 4. ***C. trachomatis*** **Immunotypes**



Highly Related: (B, Ba), (E, D), (L_1, L_2), (G, F), (C, J), (K, L_3)

SECTION II

CHLAMYDIA TRACHOMATIS INFECTIONS

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Introduction

Chlamydia organisms have a wide host range within the vertebrates — numerous species of birds and mammals, including man. The organisms of *C. psittaci* are primarily animal pathogens; those of *C. trachomatis*, primarily human pathogens. Human infections with the *C. psittaci* organisms of psittacosis — ornithosis are usually accidental infections resulting from contact with infected birds, most notably, parakeets, parrots, and turkeys.

As human pathogens, the organisms of *C. trachomatis* cause a number of diseases. Primarily these are diseases of the eye (trachoma and inclusion conjunctivitis) and of the urogenital tract (lymphogranuloma venereum (LGV), urethritis, and cervicitis). Other diseases in which Chlamydia are implicated are infant pneumonia and pelvic inflammatory disease (PID). Chlamydia may also play a role in certain other conditions in which the etiology has not been firmly established, such as Reiter's syndrome, premature birth, peritonitis, and endocarditis.

Chlamydial infections range in severity from blinding trachoma and complicated LGV to asymptomatic cases which serve as reservoirs of infection. Chlamydia organisms can coexist with other infectious agents such as *Neisseria gonorrhoeae* and be transmitted simultaneously with them.

Chlamydial infections can be treated with certain antibiotics — tetracycline, erythromycin, chloramphenicol, and sulfonamides (*C. trachomatis* infections only). Of these, tetracycline is usually the drug of choice unless contraindicated by conditions such as pregnancy. Penicillin therapy, the routine treatment for syphilis and gonorrhea, is ineffective in treating chlamydial infections.

Although infection with chlamydial organisms does stimulate antibody production, the development of protective immunity is faulty. Relapse and/or reinfection is not uncommon. In some of the diseases, trachoma for example, evidence suggests that the symptomology becomes more severe with repeated infections. Treatment also becomes more difficult. This sensitization effect has been one of the drawbacks in the successful use of vaccines as a preventive control measure.

Chlamydial infections can be subdivided into interrelated groups (Table 1) that are based on the clinical disease, the route of transmission, the distribution of the diseases, and the prevalent immunotypes. *C. trachomatis* organisms inhabit primarily the eye or the

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genital tract and can be transmitted from these sites in three ways – eye to eye, genital tract to genital tract, and genital tract to other susceptible tissue including the eye. Chlamydial diseases are distributed worldwide; however, the incidence of a specific disease may be more prevalent within certain populations as determined by geographic location and/or socioeconomic conditions. The immunotypes can be grouped and related to the various types of infection. Although these various group boundries are not always distinct, general knowledge of the groups will aid in selecting the proper line of investigation required to meet particular objectives.

Table 1. General Grouping of *C. trachomatis* Infections

Transmission	Diseases	Infected Sites	Distribution	Predominate Immunotypes
Eye to Eye	Trachoma	Eyes	Endemic: Southeast Asia, United States—Cultural Groups	A, B, Ba, C
Genital to Genital	LGV	Lymph Nodes	Tropics United States—Select Populations	L_1, L_2, L_3
	Urethritis	Urethra	Worldwide	D-K*
	Female Infections	Cervix Fallopian Tubes	Worldwide	D-K*
Genital to Other Tissue				
Adults	Inclusion Conjunctivitis	Eyes	Worldwide	D-K*
	Nonendemic Trachoma	Eyes	Sporadic, Worldwide	D-K*
Infants	Inclusion Conjunctivitis	Eyes	Worldwide	D-K*
	Pneumonia	Lungs	Probably Worldwide**	D-K*

*Most common immunotypes, ED and GF
**Data limited

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Trachoma

Clinical trachoma has been described since ancient times. It remains the world's leading cause of preventable blindness, although its incidence and areas of endemic disease are declining. Areas in which endemic trachoma is still found are the Middle East, North Africa, northern India, and Southeast Asia. Blinding trachoma can also be found in isolated areas of Latin America, Australia, and the Pacific Islands. In the United States, trachoma occurs in certain cultural and ethnic groups, such as immigrant and American Indian populations. Sporadic cases of nonendemic trachoma occur worldwide.

Clinically, trachoma is a progressive disease affecting primarily the conjunctiva of the upper eyelid. Subsequent corneal involvement may lead to blindness. Following an incubation period of perhaps 5-10 days, signs of early trachoma appear – hyperplasia and follicle formation in the conjunctiva at the edge of the upper eyelid. The cornea becomes inflamed (keratitis). As the disease progresses, the follicles swell and soften, becoming easily ruptured. Other tissues become involved and the cornea becomes vascularized as pannus formation occurs. As the disease wanes, fibrous scars form, distorting the eyelid so that the eyelashes turn inward, irritating the cornea.

The course of the disease is variable. It is influenced by the treatment regimen, by secondary bacterial infections, and by the number of previous exposures to the organism. With repeated infections, the symptomology and sequela become more severe, and disease management becomes more difficult.

Endemic Trachoma:

Endemic trachoma is a disease of hot, dry climates in areas with poor hygienic conditions. It is transmitted eye to eye by fingers, flies, and fomites and is a family disease. Children become infected in the early years and, due to constant exposure to the organism, repeated infections are common. Isolate typing and antibody evaluations show that endemic trachoma is usually immunotype A, B, B_a, or C. In the Middle East and North Africa, types A and C are predominate, whereas the B_a strain is most frequently isolated from American Indians.

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Sporadic or Nonendemic Trachoma:

Nonendemic clinical trachoma appears sporadically throughout the world. Instead of eye-to-eye transmission, the organisms are transmitted to the eye from the genital tract. Frequently an active genital infection will accompany the eye infection. Isolate typing and antibody evaluations have shown that the immunotypes are those commonly found with genital infections (types D, E, and G).

Inclusion Conjunctivitis

Studies of inclusion conjunctivitis and its relation to urogenital infections date back to the early 1900's, when inclusion bodies similar to those of trachoma were found in conjunctival epithelial cells from newborn babies suffering from nonbacterial ophthalmia neonatorum. Inclusion bodies were also found in materials taken from the urogenital tract of the infected neonates' parents. This nonbacterial conjunctivitis of the newborn was named inclusion blennorrhea.

Inclusion conjunctivitis of the newborn appears 5-12 days after birth. It is marked by a purulent exudate, but follicle formation is usually absent. In cells from conjunctival scrapings, inclusions are readily demonstrable. Although the infection will heal spontaneously in 2-3 months, it responds well to drug therapy, which is therefore recommended.

Inclusion conjunctivitis of the adult is marked by follicle formation, which is usually absent in the infant. The symptomology is much like that of trachoma but differs in that it is predominately an infection of the lower, rather that the upper eyelid, keratitis is absent, and pannus formation or scarring does not occur. It is a self-limiting disease in which the eye returns to the clinically normal state. On occasion, inflammation of the middle ear (otitis media) may accompany the eye infection.

The infectious organisms originate in the genital tract and are transmitted to the eye by various means. The newborn becomes infected in its passage through the birth canal. Incidences of infection among doctors and nurses in the field of obstetrics and gynecology indicate fingers and possibly fomites as modes of transmission. Thus, self-inoculation of the eye from one's own genital tract is not unlikely. Unchlorinated swimming pools have also been implicated in transmission of the organism. Eye-to-eye transmission is rare, but has been known to occur in those closely associated with infected newborns.

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Intermediate Syndrome

Since chlamydial eye infections vary in symptomology and severity, the clinical picture is not always typical of either trachoma or inclusion conjunctivitis but is somewhere in between. Punctuate kerato-conjunctivitis presents such a picture. For the most part, it is quite similar to inclusion conjunctivitis, but keratitis, a characteristic symptom of trachoma, is also present.

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Lymphogranuloma Venereum (LGV)

Synonyms: Climatic bubo, tropical bubo, lymphogranuloma inguinale, lymphopathia venereum, poradenitis, Durand-Nicholas-Favre disease, Frei's disease, granulomatous lymphomatosis.

LGV is a sexually transmitted disease with worldwide distribution; however, its greatest incidence occurs in regions with tropical or subtropical climates and in areas with poor socioeconomic conditions. In the United States, LGV is relatively rare, although it may be common in certain populations and/or areas.

LGV is almost exclusively a venereal disease occurring most often in sexually active adults. Infections in children are rare. Although the transmission and the site of infection is primarily genital or rectal, infections of the eye and mouth do occasionally occur through accidental contact with genital secretions.

In LGV infections, the symptomology may vary with individuals; however, the classical disease is characterized by invasion of the lymphoid tissue near the site of inoculation. As the disease progresses, other organs such as the liver, the spleen, the kidneys, and the heart may become involved as the infection becomes systemic. Complications such as elephantitis, various types of urogenital fistulas, and rectal strictures may develop. These complications can appear several years after the infection and be so severe as to require surgery.

The first clinical sign of LGV is a painless sore at the site of inoculation. The lesion appears a few days to a few weeks (usually 7-12 days) following infection. This primary lesion frequently goes unnoticed particularly if located in the female genital tract or in the area of the rectum. One week to one month later, pain and swelling in the groin occurs as the organism invades the regional lymph nodes (adenitits). These symptoms are perhaps the patient's first indication of the infection. As the disease progresses, abscesses form which drain through the skin; fever, malaise, nausea, and vomiting may occur; the infection becomes systemic, and complications may arise. Since the morbidity of the disease is related to the duration of the illness, it is important that antibiotic therapy be started as early as possible. Sexual contacts should be located and also treated. Even with treatment, relapses are common; therefore, patient surveillance should be continued for a year following an infection.

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Urethritis

Urethritis is a general term used to describe an inflammation of the urethra which is usually characterized by some sort of discharge. Although urethritis may occasionally occur in women, the primary sufferers are men. The condition may arise due to infection by a variety of organisms or to emotional stress. In many cases, a specific cause cannot be determined. Urethritis is usually categorized as being either gonococcal (*N. gonorrhoeae* organisms are present) or non-gonococcal (*N. gonorrhoeae* are absent). Nonspecific urethritis (NSU) is a term often used interchangeably with nongonococcal urethritis (NGU). For purposes of this manual, NGU will refer to all urethritis except gonococcal. Urethritis from which Chlamydia organisms are isolated is often referred to as inclusion urethritis to distinguish it from urethritis of other etiologies.

NGU is distributed worldwide and its incidence is increasing. Reports from the United States, England, Sweden, and Switzerland indicate that the incidence of NGU is rising more rapidly than that of gonorrhea. Thus NGU is probably the most common venereal disease in men.

NGU is highly infectious, with the majority of cases occurring in the 20- to 30-year age group. It has an incubation period of from 1 to 4 weeks. Symptoms may include frequent urination accompanied by pain and a mucoid or purulent (pus-containing) discharge. A heavy concentration of polymorphonuclear leucocytes (polymorphs) may be found in the urethral scrapings. With appropriate drug therapy, the symptoms abate within 2 or 3 days. Spontaneous remission will usually occur within 6 to 8 weeks. Complications that appear to be associated with NGU include epididymitis and urethral strictures; Reiter's syndrome and prostatitis have also been suggested. Recurring NGU is common; this appears to be due to reinfection rather than to relapse. Relapses, however, are common if antibiotic treatment is inadequate. Recurring urethritis is more difficult to treat and may become chronic.

In studies of NGU cases, the isolation rate of *C. trachomatis* is about 40%. Chlamydia is rarely isolated from control or normal populations. The isolation rates and the results of antibody studies indicate that Chlamydia is a causative agent of NGU. Other organisms such as *Trichomonas vaginalis, Ureaplasma urealyticum,* and *Candida albicans* may also be associated with NGU; however, Chlamydia is probably responsible for the majority of cases. Chlamydia organisms have also been shown to be closely associated

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with the occurrence of postgonococcal urethritis (PGU). This association is supported by the high rate (≥ 60%) of Chlamydia isolation from gonorrhea patients who subsequently developed PGU. Since the incubation period for Chlamydia is longer than that of gonorrhea, the two infections can be acquired simultaneously but be manifested at different times.

Infections of the Female Genital Tract

The female genital tract can be infected by a number of sexually transmitted organisms. Those commonly isolated from symptomatic cases include *Neisseria gonorrhoeae, Trichomonas vaginalis, Candida albicans*, and *Chlamydia trachomatis*. Possible sites of infection are the cervix, the vagina, the fallopian tubes, the rectum, and the urethra. Clinical symptoms are variable: inflammation, tissue change, discharge, bleeding, or pain may occur. Symptomology can rarely distinguish the infecting organism; therefore, pathological and/or laboratory testing is required for their identification. More often than not, more than one organism will be present. Thus, the etiological significance of a single agent is often difficult to establish.

Chlamydia organisms have been isolated from the cervix, rectum, and urethra of women presenting a variety of clinical symptoms. Since Chlamydia are frequently found in cases of clinical cervicitis, an etiological relationship has been suggested. The reported isolation rates of Chlamydia vary from 11% to 31%. Isolations are more frequent from women with symptomatic infections than from clinically normal women; from women who are primary (rather than secondary) contacts of men with NGU; and from women who have gonorrhea.

Chlamydia organisms can infect the genital tract of female infants during birth, resulting in a vaginitis. Genital infections have also been found in young girls up to 15 years of age with no history of sexual contact; however, these infections are usually accompanied by eye infection.

Recent data suggest Chlamydia as a possible etiological agent in pelvic inflammatory disease (PID)/salpingitis (inflammation of the fallopian tube). In cases of laparoscope-diagnosed PID, Chlamydia isolation rates of about 30% from tubal specimens have been reported. Serological studies show that high chlamydial antibody titers are present in ≥ 60% of the women with diagnosed PID.

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Chlamydia may also play a role in premature births. A high rate of prematurity has been observed among infants with Chlamydia eye infections. The ascending capability of chlamydial infections has been further supported by isolation of the organism from an infant delivered by cesarian section. Since data in this area are limited, further study is needed before conclusions can be drawn.

A number of studies attempting to relate Chlamydia isolation to various other clinical symptoms, the patient's hormonal status, the taking of oral contraceptives, and the presence of other pathogens have, for the most part, been contradictory or inconclusive.

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Infant Respiratory Tract Infections

Chlamydia is known to colonize in the eye (inclusion conjunctivitis) and vagina of infants exposed to the organisms during birth. Recent studies indicate that colonization also occurs in the respiratory tract. Chlamydia organisms have been recovered from nasopharyngeal aspirates, sputum, and lung biopsies obtained from infants with a distinctive pneumonia syndrome.

Onset of this infant pneumonia occurs in the 2nd to 3rd week of life with symptoms gradually worsening. Infants are afebrile. Tachypnea (very rapid breathing) is the outstanding feature of the infection (in some cases, it may be the only clue). Other characteristic symptoms include a distinctive cough and diffuse lung involvement. The infection remains localized in the respiratory tract and does not become systemic. Immunoglobulin (IgG and IgM) levels are elevated.

About half of the infants with pneumonia also have conjunctivitis; the other half do not. Pneumonia can develop in infants whose eye infections are treated with topical antibiotics. Pneumonia occurs in about 5% to 13% of exposed infants (an estimated 3-4 cases per 1,000 live births).

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Summary

The organisms of *C. trachomatis* are primarily inhabitants of the genital tract, but can also be found in the eye, particularly those immunotypes associated with endemic trachoma (A, B, B_a, and C). Immunotypes of genital origin (D-K, LGV 1, 2, and 3) are sexually transmitted and are chiefly pathogens of the urogenital system. The LGV types are almost exclusively genital in transmission. The types D-K inhabit the genital tract but may be transmitted to the eye or other susceptible tissue by passage through the birth canal or by finger and fomite spread. Genital infections may also ascend to involve other tissues.

For every clinically manifested infection with the genital Chlamydia, there probably exists a greater number of asymptomatic infections. Since many infections are asymptomatic or may go unnoticed, especially by women, a reservoir of infection is established which can easily be maintained in the sexually active population.

The etiological relationship of Chlamydia to LGV, trachoma, and inclusion conjunctivitis has been firmly established. Evidence now indicates that Chlamydia is one of the primary causes of nongonococcal and postgonococcal urethritis. Chlamydia has also been shown to be a cause of cervicitis, infant pneumonia, and pelvic inflammatory disease. The relationship to other disorders is less firmly established. The full significance of genital Chlamydia infections is yet to be completely understood. A fertile field for research lies ahead.

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SECTION III

LABORATORY DIAGNOSIS OF *C. TRACHOMATIS* INFECTIONS

Part A – Introduction and Direct Examination of Clinical Specimens

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Introduction

Three general approaches are used in the laboratory diagnosis of chlamydial infections:

1. Direct examination of clinical specimens
2. Isolation of the organisms
3. Detection of antibodies

These approaches vary in sensitivity, however, according to the clinical disease, the choice and handling of specimens, and the selection of the laboratory evaluation system (See Table 1). Selection of the best diagnostic method depends upon these factors and upon the capabilities of the individual laboratory.

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Table 1 Comparison of Diagnostic Methods for *C. trachomatis* Infections

INFECTION	DIAGNOSTIC METHODS							
	Direct Examination			Isolation			Serology	
	IF	Giemsa	Iodine	Cell Culture (Treated)	Eggs (ys)	Mice	Micro-IF	CF
Eye:								
Newborn	Excellent	Excellent	Good	Excellent	Excellent	– *	Good	–
Adult	Excellent	Good	–	Excellent	Good	–	Good	–
Genital:								
Urethra	Good	Fair	–	Excellent	Good	–	Good	–
Cervix	Good	Good	–	Excellent	Good	–	Good	–
Bubo	–	–	–	Excellent	Excellent	Good	Good	Good
Fallopian Tubes**				Excellent			Good	
Respiratory**				Excellent			Good	

*—Not Suitable
** Data Limited

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Direct Examination of Clinical Specimens

Direct examination of conjunctival scrapings for the presence of Chlamydia inclusions is an established method for diagnosing active eye infections. Properly performed, direct examination is as sensitive as isolation for diagnosing ocular infection in the newborn. In adult infection, the sensitivity is somewhat reduced but is still of value, particularly in patients who have been treated with antibiotics which may interfere with the recovery of the organism.

Direct examination of genital material for the diagnosis of Chlamydial infections is of limited value. In cases of urethritis, a urethral smear is routinely done to determine if the urethritis is gonococcal or nongonococcal. In the Gram-stained smear, the absence of diplococci and presence of increased number of polymorphonuclear cells ($\geq$ 4 per high-power field) indicate nongonococcal urethritis. Usually insufficient organisms are present for a diagnosis of Chlamydia. Although more Chlamydia organisms are present in cervical material than in urethral material, the presence of bacteria and mucous tend to make interpretation difficult. The nature of LGV specimens – pus, buboes – makes them unsuitable for direct examination. Therefore, for diagnosing most genital chlamydial infections, isolation is the most reliable and sensitive approach.

For direct examination, a slide is prepared from the clinical specimen, stained and microscopically examined for the presence of Chlamydia organisms and/or inclusions. Giemsa and immunofluorescent staining are the two most widely used procedures. They vary in sensitivity and applicability, however; thus, the particular procedure must be selected with care.

Giemsa Staining

Giemsa staining of clinical smears is a standard method for demonstrating Chlamydia inclusions. The technique is simple to perform, reliable, readily available, and provides permanent preparations for study. Microscopic examination of the stained preparations, however, is tedious, time-consuming, fatiguing, and requires a skilled observer for proper interpretation. Giemsa staining is most suitable for use with conjunctival scrapings from neonates, in which case it is equal in sensitivity to immunofluorescence. It is least suitable for use with urethral material.

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Immunofluorescence

Immunofluorescence (IF) is the most sensitive method available for the detection of Chlamydia in clinical specimens. IF is equal in sensitivity to Giemsa staining for evaluating conjunctival scrapings from neonates, but about twice as sensitive for evaluating adult conjunctival scrapings. It is about 4 times more sensitive for detecting Chlamydia in urethral specimens and about 1½ times more sensitive with cervical specimens. Although IF is more sensitive and the microscopy time is more rapid and less fatiguing than Giemsa, the procedure is more costly, more complex, and not readily suitable for every laboratory.

Either the direct or indirect IF procedure may be used; however, both must be carefully controlled. Each step, from the preparation of the slide to the final evaluation is critical, and must be done precisely to maintain the reliability and sensitivity of the test.

Iodine Staining

Although iodine staining is quick and easy to perform, it is not a sensitive method and not generally recommended for use with clinical material.

Papanicolaou Staining

Papanicolaou staining for detection of Chlamydia inclusions has been reported, but its use has not generally been accepted.

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Collection and Preparation of Specimens For Direct Examination

Collection

For reliable diagnosis, specimens for direct examination must contain an adequate number of epithelial cells. Therefore, to obtain the necessary cells the specimen should be collected by scraping, rather than swabbing, the infected area. For the patient's comfort, a local anesthetic can be used. Specimens should be obtained with instruments that are clean, dry, and sterile. Spatulas (blunt or sharp) are used to obtain eye specimens; spatulas or curettes are used for genital specimens. In cases of trachoma, the specimen is taken from the upper fornix of the conjunctiva; in inclusion conjunctivitis, from the lower conjunctiva. Cervical specimens are taken from the transitional zone and male urethral specimens from 3 to 6 cm into the urethra.

Slide Preparation

Clean glass slides should be used. The specimen is spread over the slide so that the cells are evenly distributed and the smear is not more than one cell thick. Ideally, at least 1,000 epithelial cells should be present for examination. The smear is air-dried at room temperature and fixed as soon as possible.

Fixation

Giemsa staining: Fix smears in absolute methanol for 5 minutes at room temperature.

IF staining: Fix smears in fresh, cold, water-free acetone for 10 minutes at 4° C. Fixed smears may be transported in a water-proof container on wet ice. However, if time in transit exceeds 2 days, the smear should be shipped and maintained in the frozen state. Repeated freezing and thawing must be avoided. Fixed slides may be stored at −20° C or, ideally, at −65° C or lower.

(See pages 57-67 for staining procedures.)

SECTION III

LABORATORY DIAGNOSIS OF *C. TRACHOMATIS* INFECTIONS

Part B — Isolation

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Introduction

Isolation of the Chlamydia organism is the most sensitive method for the laboratory diagnosis of both male and female infections of the genital tract. In cases of active eye infections, isolation is about equal in sensitivity to direct examination of eye scrapings from neonates but is somewhat more sensitive than examination of scrapings from adults.

Since Chlamydia are obligate intracellular parasites, they require a living host in which to multiply. Of the three laboratory host systems – animals, embryonated eggs, and cell culture – the cell culture is the most sensitive for isolation of *C. trachomatis*.

Of the *C. trachomatis* types, only LGV can be isolated in mice. Mice are inoculated (0.03 ml) intracranially with a diluted (10%) emulsion of the specimen. The mice become paralyzed and die within a week. At death, an impression smear is made of the dura (membrane covering the brain), which is stained and examined for the presence of Chlamydia organisms. Although mouse inoculation is an excellent method for the isolation of the agents of psittacosis and ornithosis (*C. psittaci*), it is less sensitive than cell culture and embryonated egg inoculation for the isolation of LGV. Because of its low sensitivity, mouse inoculation for isolation will not be discussed further in this manual.

All Chlamydiae can be grown in the yolk sac of embryonated chicken eggs. A major breakthrough in the study of the *C. trachomatis* strains came in 1957, when T'ang and associates were able to successfully cultivate the organisms in yolk sacs. Until cell culture techniques were developed in the early sixties, the yolk sac was the best host for *C. trachomatis* isolation. Although the yolk sac is no longer considered the choice host for isolation, it is still useful for certain research endeavors and for antigen preparation.

Growth of Chlamydia in a number of cell cultures has been reported for many years; however, use of cell culture as a host system for *C. trachomatis* isolation was unsatisfactory until the current procedures were developed. The procedures include treatment of the cells with chemicals and/or irradiation and centrifugation of the inoculum into the cells. With these procedures, cell culture is now the recommended system for isolation of *C. trachomatis*. A flow diagram for the isolation of *C. trachomatis* in cell culture is shown in Figure 1.

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It is essential that the host system, whether it be mice, eggs, or cell culture, be carefully selected. Within each type of host system, individual population differences occur that may affect Chlamydia cultivation. Differences may be due to genetic variations, or to the presence of infectious agents or inhibitory substances, but in many cases it is not known why one population is more susceptible to infection by Chlamydia than another. Therefore, only populations known to support growth of Chlamydia should be used for isolation.

Successful Chlamydia isolation requires coordination between the clinical investigators who examine the patients and collect the specimens and the laboratory that performs the cultivation procedures. Since laboratories vary in their cultivation and identification methods, specimens should be collected and transported under conditions that will complement the procedures used by the isolation laboratory and provide maximal opportunity for recovery of the organism.

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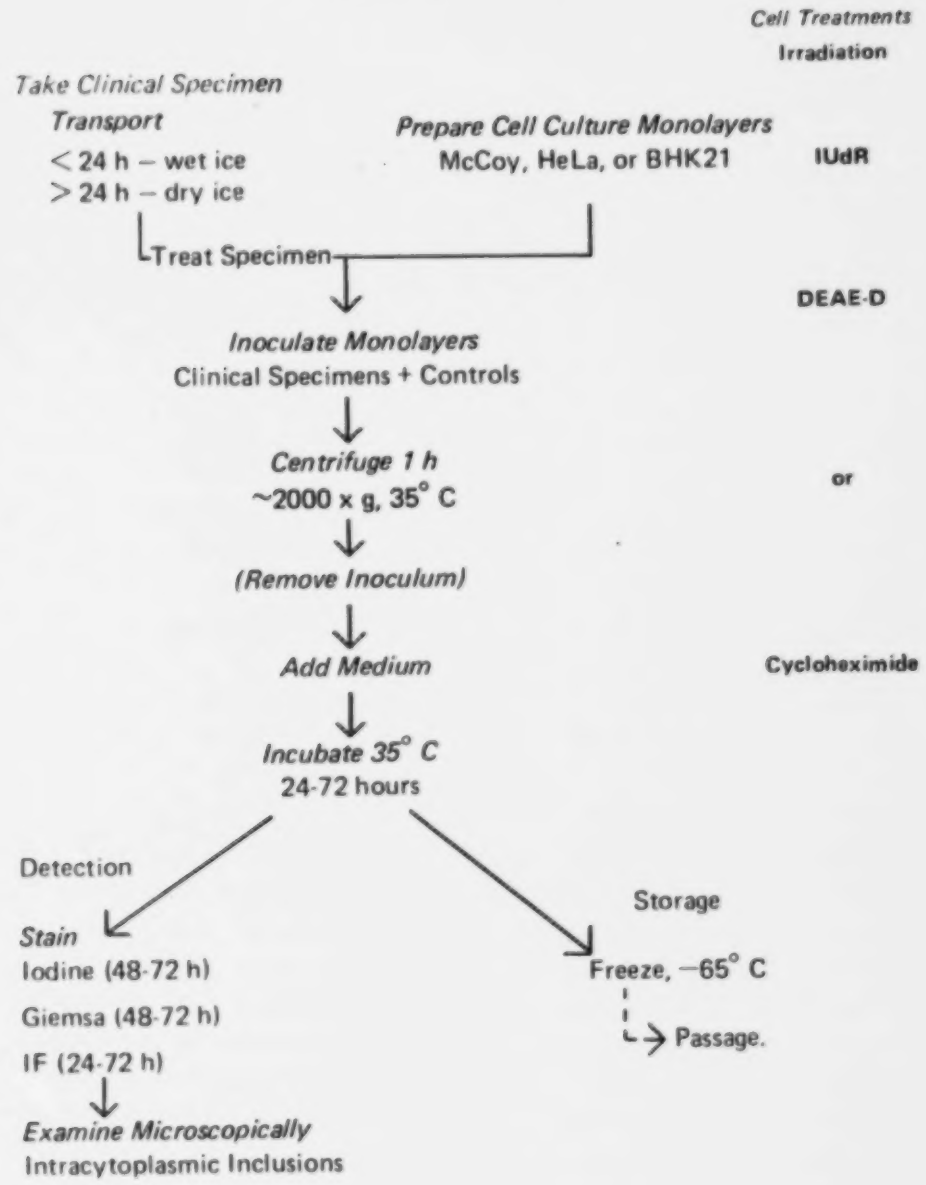
Figure 1. Flow Diagram: Isolation of *C. trachomatis* in Cell Culture



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Collection of Specimens for Isolation

In collecting specimens for Chlamydia isolation, the following factors must be considered:

1. The presence of clinical symptomology.
2. The time of collection in relation to the initiation of antibiotic therapy.
3. The site and the type of material collected.
4. The materials and reagents used for collection.
5. The temperatures used for transportation and storage of the specimen.

Presence of Clinical Symptomology

Isolation rates are higher from specimens that are taken from patients with clinical symptoms. It is rare to isolate Chlamydia from asymptomatic men, but it is not uncommon to isolate it from asymptomatic women if they are contacts of men with NGU.

Time of Collection

Specimens should be collected *before* the administration of antibiotics that are effective against Chlamydia.

Site and Type of Material

Since Chlamydia organisms can coexist with other infectious agents and can be recovered from a number of anatomical sites, it is important that when trying to establish an etiological relationship between Chlamydia and a particular disease or symptomology, the specimen be collected from the site of the infection. For example, in attempting to establish an etiologic association between Chlamydia and PID, it is more significant if the organism is isolated from a specimen taken from the fallopian tube than from the cervix.

Specimens obtained by swabbing the infected site are usually better for isolation than those obtained by scraping. The site and type of specimen to be collected for common infections follow and are summarized in Table 1.

Ocular Infections: Conjunctival swab

Cover as much of the membrane as possible without touching the skin, eyelids, or eyelashes.

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Genital Infections:

Male

Urethra: Endourethral swab

Take swab about 4 cm from the meatus. Meatal swabs, discharges, urines, and urine sediments are inferior or unsatisfactory specimens.

Epididymis: Aspirate

Take aspirate under local anesthesia by injecting sterile physiological saline and withdrawing the fluid.

Female

Cervix: Endocervical swab

Rotate swab in the transitional zone of the endocervix.

Urethra: Swab

NOTE: This site is important in the recovery of Chlamydia from female contacts of men with NGU.

Fallopian Tubes: Lumenal swab or Tissue biopsy

Take specimens during laparoscopy. Swab the lumen of the tubes from the abdominal opening. Take minute biopsy from the fimbriae (the fringed processes at the abdominal opening of the tubes).

Fluids from the pouch of Douglas are not satisfactory.

LGV: Pus and Aspirates

Aspirate pus from a fluctuant lymph node (bubo). If the bubo is not fluctuant, inject sterile saline and aspirate.

Infant Respiratory Infections: Nasopharyngeal swab, tracheobronchial aspirate, or lung biopsy.

Take swabs from the posterior of the nasopharynx. Swabs of the throat and oropharynx are not satisfactory.

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Middle Ear Infections (Otitis media): Aspirate

Obtain aspirate by myringotomy (excision of the ear drum).

Table 1. Specimens for the Isolation of *C. trachomatis*

Site	Specimen
Eye	Swab
Urethra	Swab
Epididymis	Aspirate
Cervix	Swab
Fallopian Tubes	Swab or Biopsy
Lymph Node	Aspirate
Respiratory Tract	Swab, Aspirate, or Biopsy
Middle Ear	Aspirate

Materials and Reagents

Swabs used for collection should be sterile and of a material that is not toxic to the host system or to the Chlamydia organism and does not produce artifacts when cultures are stained for evaluation. Dacron swabs may be toxic and may produce artifacts with iodine staining. Alginate swabs may produce artifacts in preparations viewed with a darkfield microscope. Many investigators use cotton swabs with satisfactory results. After collection, the swab is placed in the transport medium.

The transport medium, like the swabs, should not be toxic to the host system and should provide for survival of the Chlamydia. Suitable transport media include sucrose phosphate glutamate (SPG), cell culture growth medium containing serum (3-10%), and 0.2 M sucrose – 0.02 M phosphate (2SP) which is preferred by many laboratories. 2SP is also a suitable transport medium for the genital mycoplasmas. Adding serum, usually fetal bovine, to a 3% concentration aids in protecting the organisms from damage during transportation and storage. If serum is added, it should be ascertained to be free of Chlamydia inhibitors and antibodies. Antibiotics may also be included in the transport medium. Only antibiotics to which the Chlamydia are not sensitive should be used, for example, streptomycin, vancomycin, nystatin, gentamicin, and amphotericin B. If the specimen is also to be tested for mycoplasma, the transport medium should

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not contain antibiotics to which these organisms are sensitive. Penicillin should *never* be added to the transport medium. Transport medium is dispensed in volumes from 0.5 to 2.0 ml.

Temperatures

If the specimen is to be inoculated into the host system within 24 hours after collection, it should be kept on wet ice (4° C) without freezing. If, however, more than 24 hours will elapse before inoculation, the specimen should be quickly frozen and maintained at –65° C or lower.

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Laboratory Safety

To maintain a safe environment for laboratorians working with Chlamydia, routine saftey measures should be established and followed. Certain procedures which generate aerosols, such as sonication, and/or work with defined, highly virulent Chlamydia strains, such as highly adapted laboratory strains of psittacosis, may require additional precautions. It is important that the laboratorian be aware of the particular hazards associated with the organisms and/or the procedures that are used, so that effective safety measures can be taken.

This course has been designed and is operated with regard to the safety of each student. To protect you and your fellow students, please adhere to the safety procedures listed below and to those given orally by the instructor.

Safety Procedures

1. REPORT ALL ACCIDENTS, SPILLS, OR POTENTIAL HAZARDS TO YOUR INSTRUCTOR. He or she will know what to do.
2. Always use a pipetting device. NEVER mouth pipette.
3. Do not smoke, eat, or drink in the laboratory.
4. Practice personal hygiene. Wash your hands frequently. Keep hands away from your eyes, nose, and mouth.
5. Wear laboratory coats when working in the laboratory. If coats become soiled or contaminated, discard them in the laundry bag which will be autoclaved. Fresh coats are available. DO NOT WEAR COATS IN NONLABORATORY AREAS.
6. Wear gloves or use forceps when handling infectious, potentially infectious, and toxic material.
7. Protect your eyes from aerosols, splashes, and ultraviolet light.
8. Cover the immediate work area with absorbent toweling such as plastic backed absorbent paper. Use disinfectant soaked towels when working with highly infectious materials or when performing any procedures that produce aerosols.

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9. Wipe down the work area with a disinfectant between every procedure and at the end of each workday.

10. Do not generate aerosols unnecessarily.

11. Contain any operation that is likely to generate aerosols of infectious or toxic material. In this course, we will use the biological safety cabinet for all procedures involving infectious material.

12. Inspect all labware before use. Do not use items that are chipped, cracked, distorted, or possibly contaminated. Make certain container caps are well fitted and have a leak-proof gasket.

13. Keep all liquids tightly capped or stoppered to avoid spills.

14. Place contaminated items of general glassware in covered discard containers which will be autoclaved. Never place your hands in these containers or remove any item from them. NOTE: Special instructions will be given for disposing of certain equipment and reagents.

15. Place ONLY uncontaminated paper products in wastepaper baskets.

16. Do not discard infectious, toxic, corrosive, or explosive material in the sinks until the material has been rendered harmless by decontamination or neutralizing.

17. Be aware of potential surface contamination. Do not transfer hazardous material from a "dirty" to a "clean" surface.

18. Do not leave toxic or infectious material unattended in the laboratory. Turn off all equipment such as burners and microscopes when you leave the area, unless otherwise instructed.

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Cell Culture Systems for Isolation

Chlamydia is isolated in cell cultures which have been treated with chemicals and/or irradiated. Although a number of cell culture types have been used for growth of Chlamydia, the ones that have been established as sensitive hosts for isolation are McCoy, HeLa 229, and BHK-21 (Clone 13).

Cell Culture Types

McCoy: McCoy cells were once considered to be human synovial cells, but are now recognized to be mouse cells. Of the cell lines suitable for isolation, McCoy is the one most widely used.

HeLa 229: HeLa 229 is a cell line developed and named by W. F. Scherer in the 1950's. It presumably came from an early passage of the HeLa line established by G. O. Gey from a human cervical carcinoma.

BHK-21 (Clone 13): BHK-21 (C13) is a cloned line of the parent BHK-21 line developed in the 1960's by I. A. Macpherson and M. G. P. Stoker, from the kidneys of baby Syrian hamsters. The BHK cells that have been used successfully for Chlamydia isolation are from the cultures of the Lister Institute in London. These are often designated BHK-21 (C13) Lister.

Since individual cell populations (sublines) within each cell type vary in their sensitivity as a host system, only populations known to be highly supportive of Chlamydia growth should be used for isolation.

Untreated cell cultures are not always sensitive for Chlamydia isolation; therefore, cells are usually treated to render them more suitable for isolation. Several types of treatment have been developed.

Treatments

Irradiation: Irradiation is used with McCoy and BHK-21 cells. Confluent monolayers are exposed to ionizing radiation (5,000 rads) several days (McCoy, 3-5 days; BHK-21, 2-3 days) before they are trypsinized and the cells used to seed the culture vessels used for isolation. Irradiation prevents the cells from replicating.

IUdR (5-iodo-2'-deoxyuridine): IUdR treatment, developed for use with McCoy cells, can be used instead of irradiation. IUdR (10-25 ug/ml) is added to the cell suspension used to seed the cultures that

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will be inoculated. The cultures are incubated at least 3 days with IUdR before thay are inoculated. Cultures, however, may be used for up to 5 days after the addition of IUdR with little change in susceptibility. Before inoculation, the medium containing IUdR is removed from the cells and the cell monolayer is washed with a balanced salt solution. IUdR is an inhibitor of deoxyribonucleic acid (DNA) synthesis; thus the cells do not replicate.

DEAE-D (diethylamino-ethyl dextran): DEAE-D is used primarily for treating HeLa 229 cells but has also been used as a supplemental treatment for IUdR treated McCoy cells. Just before inoculation, a DEAE-D solution (20-30 μg/ml) is added to the cell monolayers, incubated at room temperature for 30 minutes and then removed. DEAE-D is a polycation that alters the cell membrane. Although the growth of Chlamydia serotypes A-K is enhanced by DEAE-D treatment of cells, such treatment does not affect the growth of LGV.

Cytochalasin B: Cytochalasin B, a fungal metabolite, is used similarly to IUdR. It (1 μg/ml) is added to the cell suspension used to seed the cultures that are to be inoculated and also to the medium used to maintain the cells during the growth of the Chlamydia.

Cycloheximide: Cycloheximide is used with McCoy cells. Its use eliminates the need for treating cells before inoculation. Cycloheximide (0.5-2 μg/ml) is added to the medium used to maintain the cells after inoculation. It is a glutarimide antibiotic which inhibits the synthesis of DNA and proteins in eukaryotic cells but does not affect prokaryotic cells. In cultures treated with cycloheximide, the number of Chlamydia inclusions is generally higher than in cultures treated by the other methods.

Cell Culture Requirements

A reliable source of sensitive cell cultures is essential for laboratories undertaking Chlamydia isolation. Fortunately, the commercial houses are responding to the need for Chlamydia reagents. Several companies now have McCoy cells and other reagents for Chlamydia cultivation.

Some laboratories, however, may prefer or need to culture their own cells. McCoy, HeLa 229, and BHK-21 are heteroploid cell lines and can be subcultivated indefinitely. Once a "starter" culture has

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been obtained, it can be serially cultivated with relative ease. Techniques for cell cultivation can be found in the Centers for Disease Control (CDC) manual, *Basic Laboratory Techniques in Cell Culture.*

The cell cultures and media used for Chlamydia must be kept free of contaminating organisms, including mycoplasma. Ideally, stock cultures should be maintained on antibiotic-free media and antibiotics added only to those cultures used for isolation. The antibiotics are usually the same as those used in the transport medium.

A modified Eagle's minimal essential medium (MEM) is used both for growing the cells and maintaining them after inoculation with Chlamydia. Extra glucose (twice the normal amount required by the cells) is added to the medium to enhance chlamydial growth. If BHK-21 cells are used, tryptose phosphate broth is added to the medium to a 10% concentration. For cell growth, serum is required. The serum (usually fetal bovine) is heat-inactivated (56°C/30 minutes) and is added to the medium to a 10% concentration. Any serum used should be pretested and ascertained to be free of inhibitors, microorganisms, and Chlamydia antibodies. Serum (5% – 10%) is usually also incorporated into the medium used to maintain the cultures after they have been inoculated. HEPES [4 – (2 – hydroxyethyl) – 1 – piperazine – ethanesulfonic acid] buffer may also be added to this medium to help control pH.

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Preparation of Cell Culture Monolayers for Chlamydia Cultivation

Materials

Confluent Cell Line Monolayer

Trypsin, 0.2%

Growth Medium – Eagle's MEM containing 10% fetal bovine serum

Trypan Blue, 0.4%

Cell Counting Chamber and Cover Slip

Hand Tally Counter

Shell Vials, 1 dram containing a round cover slip, 12 mm.

Vial Closures, plastic caps or stoppers

Procedures

Part I – Monolayer Trypsinization

NOTE: If irradiation is being used to treat the cells, it is done 2-5 days, depending on cell type, before the monolayer is trypsinized.

1. Decant the medium from the monolayer culture.
2. Wash the monolayer with a small amount of trypsin (2-3 ml/25 cm^2 flask). Phosphate buffered saline (PBS), Mg++ and Ca++ free, may be used for washing instead of trypsin.)
3. Add trypsin in a volume sufficient to cover the monolayer (2-3 ml/25 cm^2 flask).
4. Incubate the culture at room temperature with the trypsin in contact with the cells for 1-2 minutes (McCoy cells come off quickly – watch them carefully). NOTE: If cells detach during this incubation, quickly remove the trypsin. Detached cells may be recovered by centrifuging the trypsin/cell mixture.
5. Remove and discard the trypsin.
6. Incubate the culture with the cell sheet down at room temperature or 37° C until the cells detach and separate. Determine detachment by holding the culture to the light and observing the cells.

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7. Add growth medium (7 ml/25 cm^2 flask) and suspend the cells by pipetting.

Part II Cell Counting

1. Clean the counting chamber and cover slip with water and then with alcohol. Dry both thoroughly. Lightly moisten the supporting ridges of the chamber and apply the cover slip.

2. Mix the cell suspension and asceptically remove a 0.5-ml sample. Combine the sample with 0.5 ml of trypan blue. This results in a 1:2 dilution of the cell suspension.

3. With a capillary pipette, carefully fill one side of the counting chamber. Do not allow the fluid to overflow.

4. Examine the cells through a microscope with the 10X objective. Count the unstained cells in 4 large squares (4 corner squares). See Figure 2.

   NOTE: The living cells are unstained and the dead cells are stained blue. Cells lying on the top and left side lines of each square are omitted from the count, but cells on the bottom and right side lines are included. Count each cell in clumps of cells, but if too many clumps are present or if the clumps are large, the count will not be reliable.

5. Using the following formula, estimate the number of cells per milliliter of the original suspension.

$$\frac{\text{No. Cells Counted}}{\text{No. Squares Counted}} \times \text{Counting Chamber Conversion Factor} \times \text{Dilution Factor} = \text{Cells/ml}$$

Hemocytometer Conversion Factors:

Spencer Bright-Line, 0.1 mm deep = 10,000
Fuchs-Rosenthal, 0.2 mm deep = 5,000

Example:		
	Total number of cells counted	100
	Number of squares counted	4
	Hemocytometer Depth	0.1 mm
	Dilution Factor	2

Thus:

$$\frac{200}{4} \times 10{,}000 \times 2 = 1{,}000{,}000 \text{ cells/ml}$$

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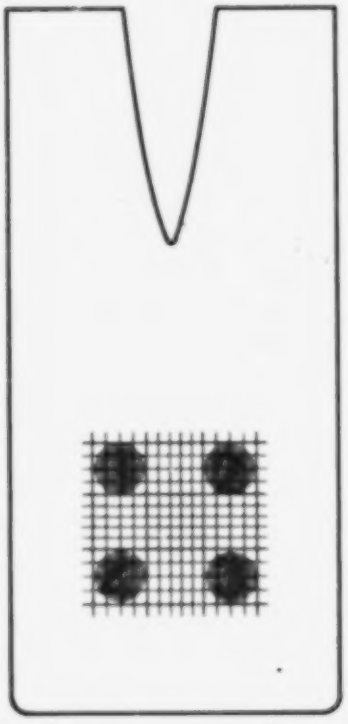
Figure 2. Cell Counting Chambers



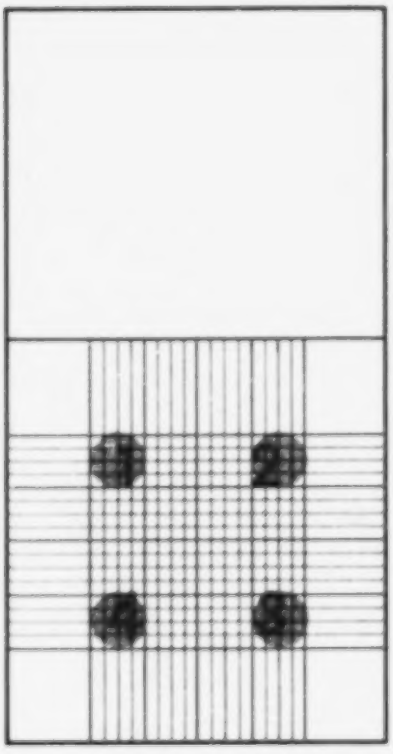
SPENCER BRIGHT LINE

CONVERSION FACTOR: 10,000

BASIS OF THE CONVERSION FACTOR:

VOLUME = 1 mm (side) x 1 mm (side) x 0.1 mm (depth) = 0.1 mm^3
1 mm^3 = 10 x 0.1 mm^3
1 cm^3 = 1000 x 1 mm^3
1 cm^3 is approximately 1 ml

THE NUMBER OF CELLS CONTAINED IN 1 ML IS 10,000 TIMES THAT WHICH IS CONTAINED IN 0.1 mm^3.



FUCHS-ROSENTHAL

CONVERSION FACTOR: 5,000

BASIS OF THE CONVERSION FACTOR:

VOLUME = 1 mm (side) x 1 mm (side) x 0.2 mm (depth) = 0.2 mm^3
1 mm^3 = 5 x 0.2 mm^3
1 cm^3 = 1000 x 1 mm^3
1 cm^3 is approximately 1 ml

THE NUMBER OF CELLS CONTAINED IN 1 ML IS 5,000 TIMES THAT WHICH IS CONTAINED IN 0.2 mm^3.

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Part III – Dilution of the Cell Suspension

1. Using the following formula, determine the amount of cell suspension and growth medium required to give the desired cell concentration and volume.

   (For class purposes _____ ml of _____ cells/ml is needed.)

   Formula: $C_I V_I = C_F V_F$

   C_I = Concentrate (Initial Concentration)

   V_I = Volume of Concentrate (Initial Concentration)

   C_F = Desired Concentration (Final)

   V_F = Desired Volume (Final)

   Example: C_I = 2,500,000 cells/ml

   V_I = Unknown volume of initial concentrate

   C_F = 150,000 cells/ml

   V_F = 50ml

   $$2{,}500{,}000\ V_I = 150{,}000 \times 50$$

   $$2{,}500{,}000\ V_I = 7{,}500{,}000$$

   $$V_I = 3 \text{ ml}$$

   Thus: 3 ml of the original suspension is combined with 47 ml of growth medium to give 50 ml of suspension with a concentration of 150,000 cells/ml.

2. Using the results of the calculations above, dilute the original suspension to give the desired final concentration and volume. NOTE: If cells are to be treated with IUdR or cytochalasin B, it is added at this time.

Part IV – Seeding the Culture Vessels

The culture vessels (vials) referred to throughout this manual are 1 dram shell vials containing a 12 mm round glass cover slip. Plastic threaded push-in caps, which are airtight, are used as closures. See Figure 3.

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1. Keeping cells well suspended, add 1 ml of cell suspension to each vial.

2. Securely cap each vial and label vials with the cell type and date.

3. Incubate cultures at 37° C.

4. Monitor cultures daily. The monolayers can be examined with an inverted microscope and should be confluent (See Figure 4.) in 2-3 days. Do not use cultures that are cloudy, alkaline, or contaminated.

Comments:

Vials can be seeded with varying concentrations of cells to provide monolayers over a period of several days.

Nontoxic rubber or silicone stoppers can also be used as closures for the vials.

For microculture, 5mm round cover slips are placed in 96-well flat bottom microculture plates. Each well of the plates is seeded with 0.2 ml of cell suspension. Since the lids of the microculture dishes do not provide an airtight closure, cultures must be incubated in a humid atmosphere of CO_2 (~5%) to maintain pH. Note: Some investigators feel that cross-contamination between wells of the microculture dish can occur.

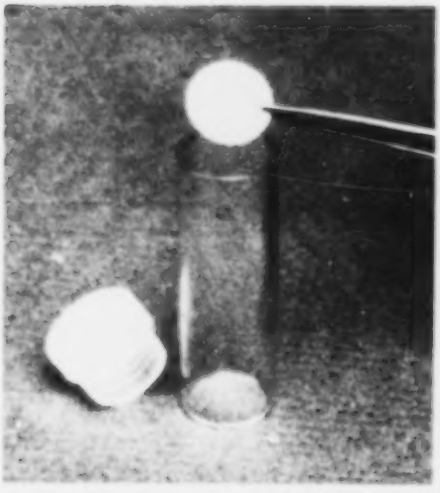

Figure 3. One Dram Shell Vial, 12mm Round Glass Cover Slip, and Plastic Cap

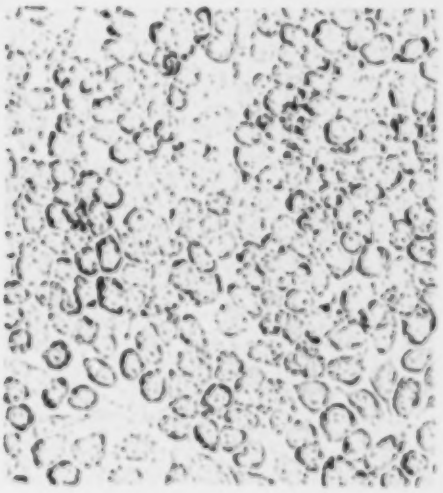

Figure 4. Confluent McCoy Cell Monolayer

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Inoculation of Cell Cultures with Unknown Specimens

Materials

Confluent Cell Monolayers on Cover Slips in Culture Vials

Positive Control Culture (Produces a known number of inclusions)

Negative Control (Transport medium)

Unknown Specimens

Culture Medium – Eagle's MEM containing 10% fetal bovine serum and HEPES buffer

Cycloheximide Stock Solution (400μg/ml)

Procedures

Part I – Preparation of Specimens

Specimens in transport medium (swabs, aspirates, or scrapings): Frozen specimens are quickly thawed by agitating them in a 37° C water bath. To enhance suspension of the organism, the specimen can be agitated with a mixer, shaken with glass beads, or sonicated.

Bubo Pus: Viscous material is ground and suspended to a 20% w/v in transport or cell culture medium. Antibiotics are added to eliminate contaminants. Dilution of the suspension is often required to reduce toxic substances.

Biopsy Tissue: Tissue is ground and suspended to a 20% w/v in transport or cell culture medium. Antibiotics are added to eliminate contaminants. Holding the suspension at 4° C for 18-24 hours enhances the diffusion of the organism into the medium and allows for sedimentation of tissue. A light centrifugation (200 x g, 5 minutes) may be used when additional clarification is needed.

A sample of the specimen (approximately one-half) should always be retained as a safety measure and as needed for additional study. It should be quickly frozen (dry ice and alcohol or acetone) and stored at –65° C or lower.

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Part II – Inoculation of Cell Cultures

1. Microscopically examine the cell cultures to ascertain that the monolayers are confluent and free of contaminants.

2. Label the cell culture vials as follows:

   3 vials for *each* unknown specimen

   3 vials for the positive control

   3 vials for the negative control

   2 vials as uninoculated controls (optional)

   NOTE: Use of 4 rather than 3 cultures may slightly increase the chance of isolation on the first attempt.

3. Remove the growth medium from the cultures.

   NOTE: Following removal of the growth medium, cells treated with IUdR are washed with a balanced salt solution (BSS) such as Hanks'. If DEAE-D treatment is being used, 1 ml of Hanks' BSS containing 20-30 ug/ml DEAE-D is added to each monolayer. After a minimum of 30 minutes incubation at room temperature, the DEAE-D solution is removed and the monolayers inoculated.

4. Inoculate 0.1ml of the positive control, the negative control, and the unknown specimens into each of the corresponding prelabeled vials.

5. Centrifuge the inoculated cultures for 1 hour at 33° – 35° C at as high a speed as can be safely obtained with a conventional laboratory centrifuge, but not at a speed below 700 x g. See Figure 5.

   NOTE: For routine isolation, centrifugation at 2,500 to 3,000 x g for 1 hour at 33° – 35° C is recommended; however, some laboratories have had satisfactory results with speeds as low as 700 x g. In a study using irradiated McCoy cells (Darougar, 1974), centrifugation at 15,000 x g for 1 hour at 35° C was found to be optimal. The interior and contents of an insulated centrifuge will usually reach the desired temperature from the heat generated by the operation of the machine at the speeds required for Chlamydia. The centrifuge can be prewarmed by running it *unloaded* at its maximum speed and with no refrigeration. Hot air blowers (hair dryers) can also be used for prewarming.

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6. During the centrifugation, prepare the culture medium as follows:

   a. Measure the culture medium in a graduated cylinder. Record the volume _____ ml.

   b. Add sufficient stock solution of cycloheximide to the culture medium to obtain a final concentration of _____ µg/ml.

      NOTE: Individual lots of cycloheximide vary. It is necessary to titer each lot to determine the optimal concentration for Chlamydia growth.

   c. Place the culture medium containing the cycloheximide in the refrigerator until it is needed. (Be sure to label it with your name.)

7. Remove the inoculum.

   NOTE: This step aids in removing possible toxic material and may not be needed for all preparations. Those preparations most likely to be toxic include materials suspended in SPG, LGV specimens, tissue suspensions, and multiple-passaged laboratory preparations. Rinsing of the cell monolayer with cell culture medium may further aid in reducing toxicity.

8. Add 1 ml of culture medium containing cycloheximide to each vial.

9. Incubate the cultures at 35° C for 48 – 72 hours before staining. If immunofluorescence procedures are being used for detection, cultures may be stained after 24 hours' incubation.

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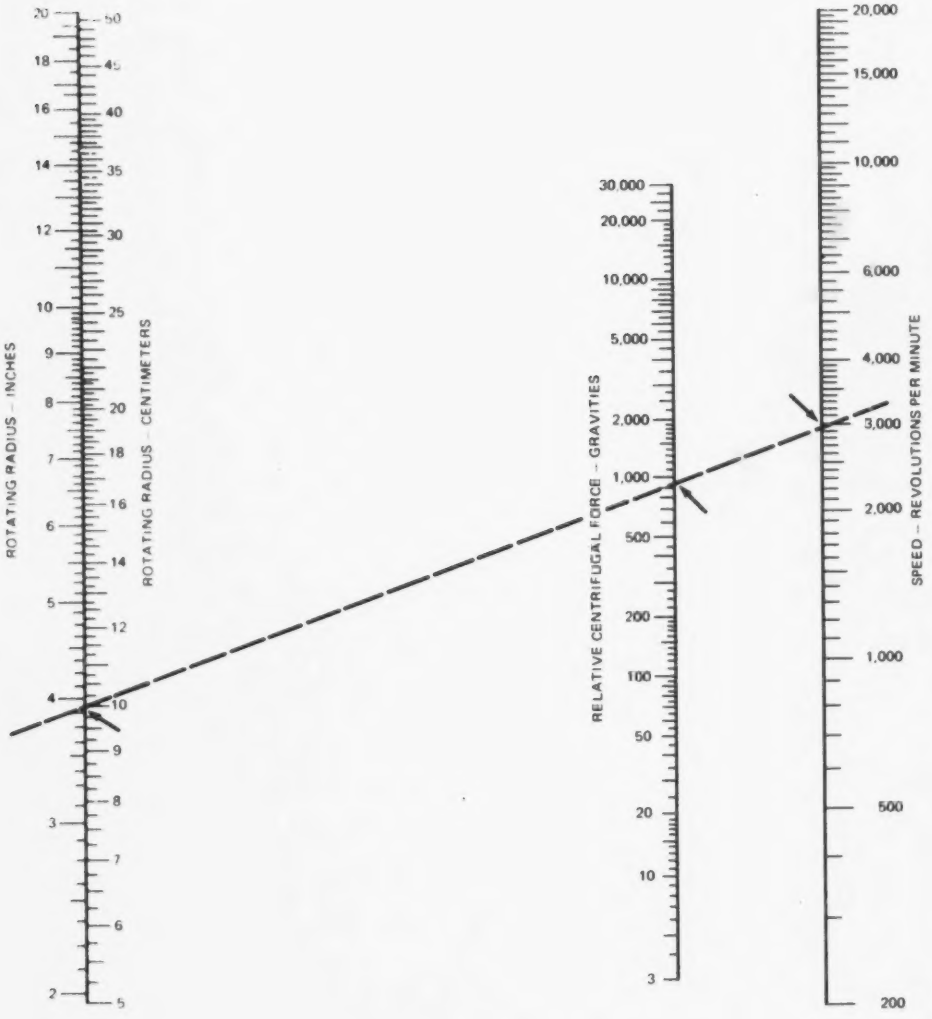
Figure 5. Nomograph for Calculating Centrifuge Speed


ROTATING RADIUS – INCHES
ROTATING RADIUS – CENTIMETERS
RELATIVE CENTRIFUGAL FORCE – GRAVITIES
SPEED – REVOLUTIONS PER MINUTE

EXAMPLE:
To find the speed (revolutions per minute) place a straightedge on the chart connecting the appropriate point on the Rotating Radius Scale (for example, 10 cm)* with the appropriate point on the Relative Centrifugal Force Scale (for example, 1,000 X g). Read the point at which the straightedge intersects the Speed Scale (3,000) as the desired revolutions per minute.

*To determine the rotating radius of the centrifuge head, measure – with the tube in the extended position – from the center of the drive shaft to the middle of the liquid column you are centrifuging.

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Staining Reactions

The Chlamydiae are basophilic in nature and have an affinity for basic dyes. Chlamydiae are classified as Gram-negative, but the Gram stain is of little practical use because the staining reaction is so variable. There are, however, a number of stains that can be used successfully to detect Chlamydiae inclusions and elementary bodies in the cells of clinical smears and laboratory hosts. The stains most commonly used are described below.

Giemsa:

Giemsa is the "classic" stain for demonstrating the presence of Chlamydia. It can be used for detecting chlamydial inclusions in clinical material and in laboratory hosts. Giemsa stains all the developmental forms of the organisms of both species, but will not differentiate between species. Although Giemsa is not a Chlamydiae-specific stain, Chlamydiae can be distinquished from other organisms by the color reaction, morphology, and location of the inclusion body.

May-Grünwald-Giemsa:

The staining reaction with May-Grünwald-Giemsa is similar to that of Giemsa.

Iodine:

Iodine is a selective stain for glycogen; therefore, its use is limited to the *trachomatis* species which produces glycogen. Since glycogen is present only during certain phases of the developmental cycle, iodine staining is not as sensitive a method for demonstrating chlamydial inclusions in clinical material as is Giemsa staining. Use of iodine is of some, but limited, value in the evaluation of eye scrapings, but of no value in the evaluation of cervical material since normal cervical cells contain glycogen. Of great value, however, is the use of iodine for the staining of timed cell culture preparations because it provides a quick and reliable method for the detection of *C. trachomatis* infected cells.

Giménez (Basic Fuchsin and Malachite Green):

Giménez stain detects Chlamydia elementary bodies. It is used primarily to evaluate laboratory preparations in which the cells are ruptured, for example, yolk sac smears, fluid suspensions, and impression smears from animal hosts. Giménez staining provides excellent contrast between the host tissue components (which stain green)

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and the elementary bodies (which stain red). Giménez is frequently used when evaluating the infectivity of a preparation.

Macchiavello (Basic Fuchsin and Methylene Blue):

Macchiavello staining is used similarly to Giménez staining but the contrast between the organisms and the host tissue is not as good.

Acridine Orange:

Acridine orange is a fluorochrome. When stained preparations are examined under ultraviolet light, cellular DNA appears yellow-green and RNA, red. Although not used routinely for diagnostic work, this dye is useful for studying the developmental cycle.

Immunofluorescent Stains (Conjugates):

Conjugates (antibodies labelled with fluorochromes) vary in composition and quality. High-quality conjugates used in either the direct or the indirect immunofluorescence staining procedure can provide both a specific and a sensitive means for the detection and identification of Chlamydia in cells of laboratory hosts and clinical specimens. Immunofluorescence staining detects Chlamydia antigens throughout the developmental cycle and is not selective for the infective particles.

The stains described above vary in specificity and sensitivity. When selecting the stain to use, it is important that the organism's species, the phase of the developmental cycle, the type of preparation, and the investigational purpose all be taken into account.

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Giemsa Staining

Materials

Slide or Cover Slip Preparations:

Positive Control

Negative Control

Unknown Specimens

Hanks' BSS

Methanol, Absolute

Giemsa Stain (Stock Solution)

Distilled Water or Phosphate Buffer

Ethanol, 95%

Xylene

Mounting Resin (Permount)

Procedure

This procedure describes the staining of cover slips in the original culture vials.

1. Prepare the working solution of stain by diluting the stock Giemsa_____in distilled water. The working solution is prepared fresh daily.

   NOTE: The dilution used to prepare the working stain may vary with the stock preparation and the time and temperature of the staining procedure. Usually dilutions in the range of 1:20 to 1:25 will give satisfactory results. Phosphate buffer may be used for diluting the stain.

2. Remove the media from the cultures.
3. Gently rinse the monolayers with Hanks' BSS (1 ml).
4. Add 1 ml of absolute methanol to each vial. Fix for 10 minutes.
5. Remove the methanol and add 1 ml of *diluted* Giemsa stain.
6. Stain for a minimum of 1 hour. Staining may be enhanced by incubation at 35° C and/or for a longer period (1–1½ hours).

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7. During the incubation, clean and appropriately label microscope slides to correspond to each cover slip being stained.
8. Remove the stain and rinse the preparation for a few seconds in 95% ethanol.
9. Place 1 drop of mounting medium on a clean, prelabeled slide.
10. Remove the cover slip from the vial that corresponds to the label on the slide. Dip the cover slip into xylene and drain off the excess. Gently lower the cover slip, *cell side down*, onto the drop of mounting medium.

    NOTE: If slide preparations of clinical material are being stained, they are air dried after step 8 (rinsing in ethanol). They can be examined without mounting.
11. Repeat steps 8 and 9 until all the cover slips are mounted.
12. With a high-power objective, microscopically examine the stained cells for the presence of intracytoplasmic inclusions characteristic of Chlamydia. Examine questionable inclusions with oil immersion.

Interpretation: See Figure 6. Both brightfield and darkfield illumination can be used to examine Giemsa-stained preparations. With brightfield, the cell nuclei are reddish purple and the cytoplasm, bluish purple; the Chlamydia elementary bodies are red to purple and the reticulate bodies, blue to purple. With darkfield, the particles within Chlamydia inclusions fluoresce bright golden yellow; however, the background frequently contains substances that also fluoresce. In cell culture preparations the nonspecific fluorescence is usually white or blue and can usually be distinguished from Chlamydia fluorescence. Clinical specimens, on the other hand, often contain cells, bacteria, and other substances that fluoresce. Because of the amount of nonspecific background fluorescence, the use of darkfield illumination with clinical specimens is limited.

Comment: The Giemsa staining procedure is a reliable method for the detection of inclusions of both the Chlamydia species. Giemsa staining is easy to perform, suitable for use with clinical specimens, can differentiate the elementary and reticulate bodies, and is permanent. Examination of the stained preparations, however, is often difficult, requiring an experienced microscopist. This procedure can be quite fatiguing and time-consuming, requiring 30 minutes to 1 hour per preparation.

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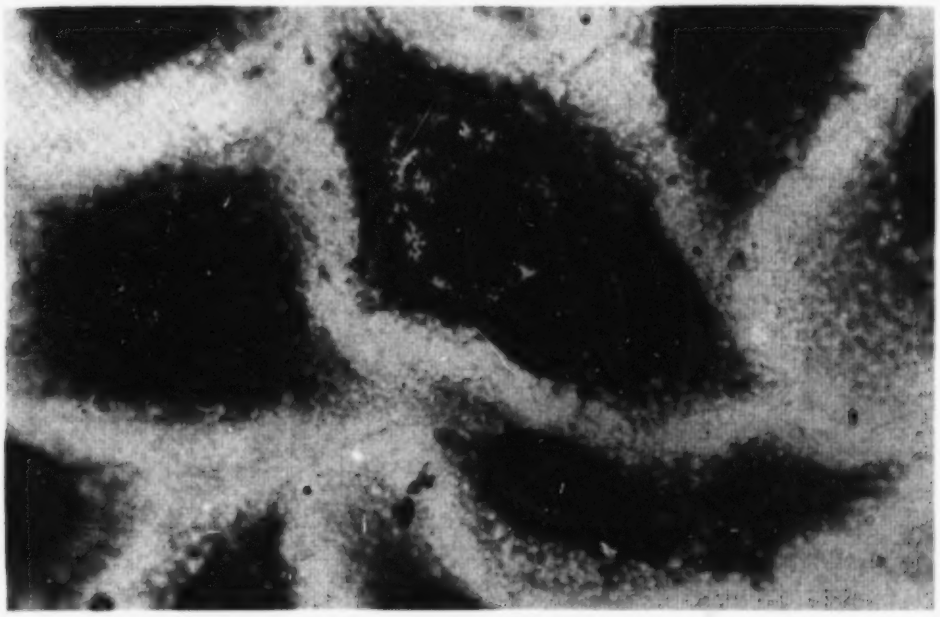
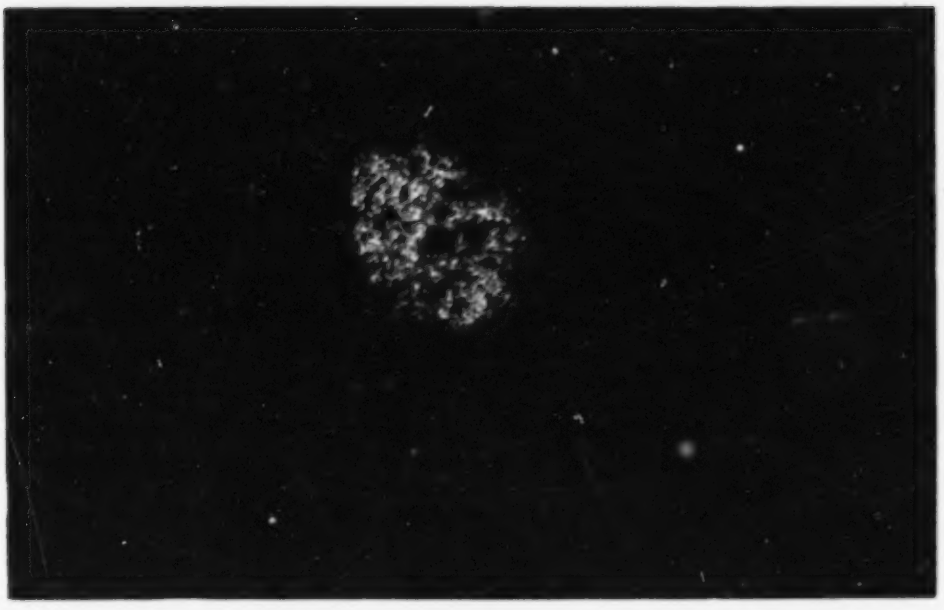


a. Brightfield



b. Darkfield

Figure 6. Infected McCoy Cells, Giemsa-Stained

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Iodine Staining

Materials

Slide or Cover Slip Preparations:

- Positive Control
- Negative Control
- Unknown Specimens

Hanks' BSS

Fixative Solution (Methanol/Formalin)

Methanol, Absolute

Jones' Iodine Stain

Glycerol

Procedure

This procedure describes the staining of cover slips in the original culture vials.

1. Filter the iodine stain through a Whatman #41 ashless paper. The stain is filtered each time it is used.
2. Prepare the mounting medium by combining equal parts of filtered iodine stain and glycerol (5 ml + 5 ml). The mounting medium (iodine/glycerol) is for use throughout the course.
3. Remove the media from the cultures to be stained.
4. Gently rinse the cell monolayers with Hanks' BSS (1 ml).
5. Add 1 ml of fixative solution to each vial. Let stand for a minimum of 10 minutes and then remove.
6. Add 1 ml of absolute methanol to each vial and let stand for a minimum of 10 minutes before removing.
7. Add 0.5 ml of filtered iodine stain to each vial. Let stand for 10 minutes and then remove.

NOTE: During the incubation periods, clean and appropriately label microscope slides to correspond to the cover slips being stained.

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8. Place 1 drop of iodine/glycerol mounting medium on a clean, prelabeled slide.

9. Remove the cover slip from the vial that corresponds to the label on the slide and gently lower it (*cell side down*) onto the drop of mounting medium.

10. Repeat steps 8 and 9 until all the cover slips are mounted.

11. Using a light microscope, examine the stained cells with low power for the presence of brown staining inclusions. Confirm inclusions by examining them under high power to ascertain that they are intracytoplasmic and characteristic of Chlamydia.

Interpretation: See Figure 7. The intracytoplasmic inclusion bodies formed by *C. trachomatis* contain glycogen between 24 to 72 hours after inoculation. When stained with iodine, the glycogen-containing inclusions stain dark to reddish brown against the yellow background of the cells. In some inclusions, granularity will be seen. Suspected Chlamydia inclusions should always be confirmed under high magnification to ascertain that the stained masses are intracytoplasmic and typical of Chlamydia inclusions. Artifacts that stain brown and lie on top of cells may resemble Chlamydia inclusions and may cause problems with interpretation. (See Figure 8.)

Comment: Iodine staining is easy to perform and the darkly stained *C. trachomatis* inclusions are easily seen. Use of iodine stain is a reliable method for detecting Chlamydia infected cells in certain types of cell cultures if the staining is timed appropriately with the organism's developmental cycle. Iodine staining is excellent for use with McCoy cells and preferred by most laboratories using McCoys. With other cell types, the staining reaction is not always satisfactory, thus laboratories using HeLa 229 or BHK-21 cells usually use Giemsa or IF procedures.

NOTE: Preparations stained with iodine may be decolorized with methanol and restained with Giemsa for confirmation or to overcome difficulties in interpretation.

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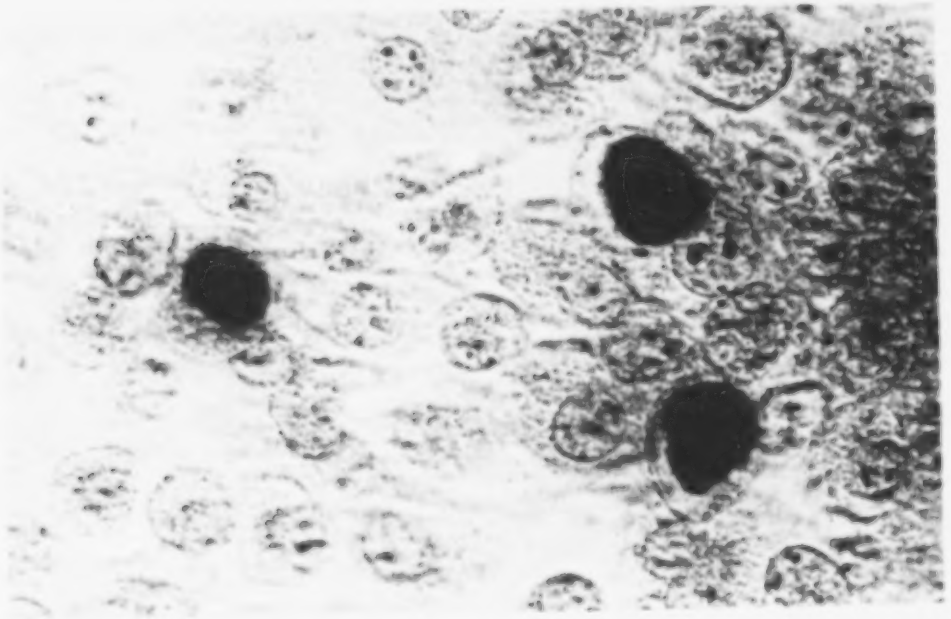

Figure 7. Infected McCoy Cells, Iodine-Stained

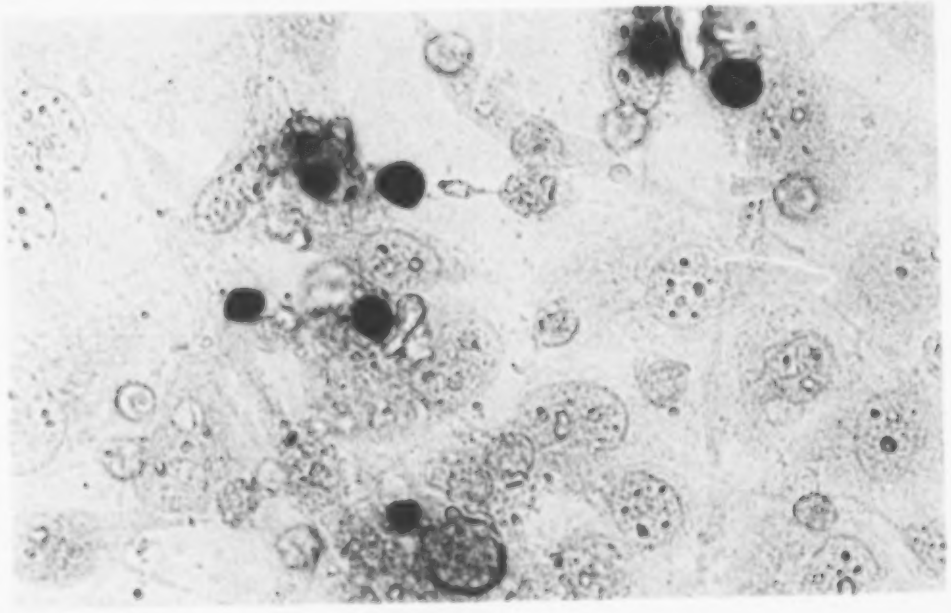

Figure 8. Artifacts: Iodine-Stained McCoy Cells

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Immunofluorescence Staining: Direct and Indirect Tests

Materials

Slide or Cover Preparations:

- Positive Control
- Negative Control
- Unknown Specimens

Acetone, Water-free, cold

PBS, pH 7.2 – 7.4

Glycerol Mounting Medium

For Direct Test:

- FITC Conjugate, fluorescein-labeled anti-Chlamydia (LGV), pretitered

For Indirect Test:

- Chlamydia antiserum, hyperimmune rabbit or human convalescent (LGV), pretitered
- FITC Conjugate, fluorescein-labeled goat antirabbit or antihuman, pretitered

Procedures

These procedures describe the staining of cover slips in the original culture vials.

Fixation:

1. Remove the medium from the inoculated cultures.
2. Gently rinse the cell monolayers with PBS (1 ml).
3. Add 1 ml of cold, water-free acetone to each vial and then remove.
4. Again, add 1 ml of acetone to each vial. Securely stopper each vial with a rubber stopper, size 0.
5. Fix cells for 10 minutes at 4° C.

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6. Remove the acetone and allow the cover slips to air-dry. Fixed, dried cover slips can be stored at 4° C for staining the following day or for extended periods at −65° C.

Direct Test:

1. Add 0.2 ml of Chlamydia FITC conjugate to each vial containing a dried, fixed cover slip. (It is important that the cells are completely dry before staining.) Securely stopper each vial with a rubber stopper.
2. Incubate the stoppered vials at 37° C for 30 minutes. (A moist environment must be maintained throughout the staining process to prevent drying of the reagents.)
3. Remove the conjugate.
4. Add at least 1 ml of PBS to each vial. Securely stopper and place vials in a rack anchored on an agitator such as the kind used for serological tests. Agitate for 10 minutes.
5. Remove the wash fluid (PBS) and repeat step 4 (Total: 2-10 minute PBS washes). Preparations may be counterstained by adding a few drops of Evans Blue (1%) or Trypan Blue (0.25%) to the PBS in the final wash.

   NOTE: During the incubation periods, clean and appropriately label microscope slides to correspond to each cover slip being stained.
6. Remove the final PBS wash and rinse the cover slips with distilled water.
7. Place 1 drop of glycerol mounting medium on a clean prelabeled slide.
8. Remove the cover slip from the vial that corresponds to the labeled slide and gently lower it (*cell side down*) onto the drop of mounting medium.
9. Examine the stained preparations with a fluorescence microscope.

Indirect Test:

1. Add 0.2 ml of Chlamydia antiserum to each vial containing a dried, fixed cover slip. Securely stopper each vial with a rubber stopper.

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2. Incubate the stoppered vials at 37° C for 30 minutes.
3. Remove the antiserum.
4. Wash the preparations in 2 changes of PBS for 5 minutes each. Use the procedure described in the direct test. Remove the wash fluid.

   NOTE: When staining slides that have more than one antigen or serum dilution, for example, in the micro-IF test, the preparations should be air dried before the addition of the conjugate.
5. Add 0.2 ml of antispecies conjugate to each vial. Securely stopper the vials.
6. Incubate the stoppered vials at 37° C for 30 minutes.
7. Remove the conjugate.
8. Wash the stained preparations in 2 changes of PBS for 10 minutes each as previously described. Cells may be counter-stained as in the direct test.
9. Remove the PBS and rinse the cover slips in distilled water.
10. Mount the cover slips on clean, labeled microscope slides as described in the direct test.
11. Examine the stained preparations with a fluorescence microscope.

Interpretation: See Figures 9 and 10. Chlamydia inclusions are seen as brightly fluorescing yellow masses in the cytoplasm of epithelial or cell culture cells near the nucleus. Clinical specimens are regarded as negative if a sufficient number of cells is present and typical fluorescence is not observed.

Comment: Non-Chlamydia fluorescence can occur and it must be recognized as such. If the conjugate has not been adequately diluted, it will often stain nonspecifically. Components of the smear other than Chlamydia infected cells may also stain – polymorphonuclear leucocytes, lymphocytes, bacteria, clumped-pigment granules, and keratinized cells. Stain may be trapped in folded cells or may accumulate in areas that dried during staining.

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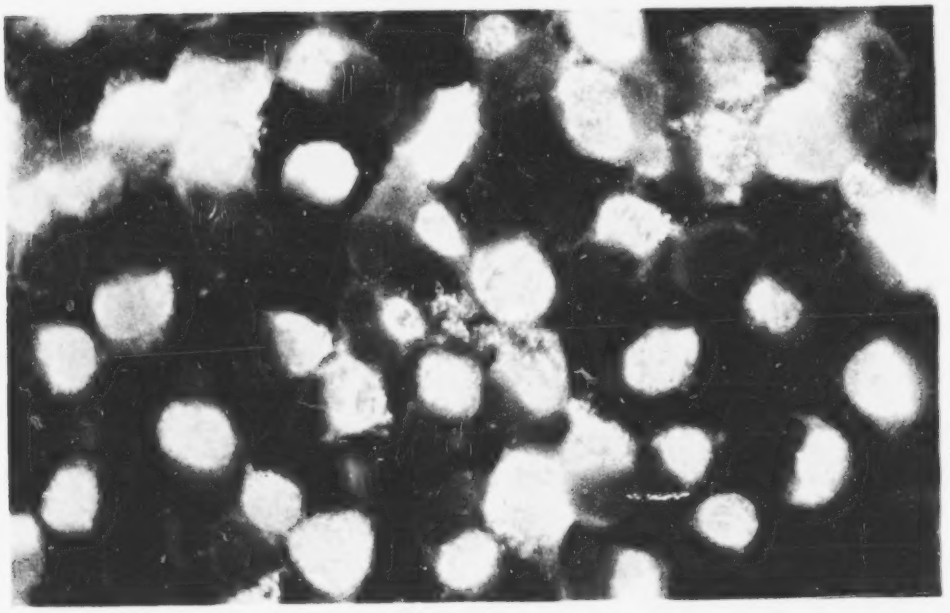

Figure 9. Heavily Infected McCoy Cells, Stained by Indirect IF

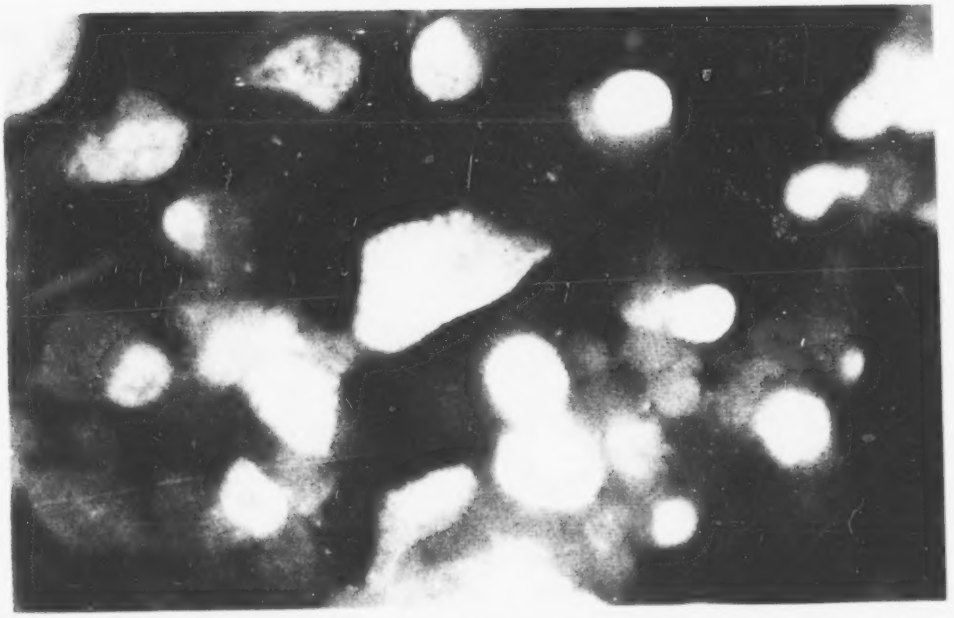

Figure 10. Heavily Infected BHK-21 Cells, Stained by Direct IF, Counterstained with Evans Blue

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Giménez's Staining

Materials

Smears, gently heat fixed (optional)

Carbol Fuchsin, filtered working solution

Malachite Green solution

Procedure

1. Cover the smear with the filtered basic fuchsin solution for 1-2 minutes.
2. Rinse in tap water.
3. Cover the smear with malachite green solution for 6-9 seconds.
4. Rinse in tap water.
5. Repeat steps 3 and 4 if necessary.
6. Blot smear with absorbent paper.
7. Examine the stained smear under oil immersion for the presence of elementary bodies.

Interpretation: The Chlamydia elementary bodies stain red and the reticulate bodies and host cell components stain green. This stain is used most frequently for evaluating yolk sac smears and mouse dura impressions. In these preparations, the cells are usually ruptured, thus the elementary bodies are free in the cellular material and are not contained in an inclusion.

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Evaluation of Inoculated Cultures

Chlamydia growth in cell culture is usually limited to one developmental cycle. For each infectious particle (EB) that enters the cell, an inclusion is formed in the cell's cytoplasm. A cell can be infected with more than one EB and thus may have more than one inclusion. The developmental cycle takes place within the inclusion(s), and at completion, the cell ruptures liberating the newly formed EBs. These EBs, however, do not infect other cells.

Chlamydia organisms, with some exceptions, do not produce any readily visible cytopathic effect (CPE). Infected cultures are evaluated by examining stained cells for the presence of intracytoplasmic inclusions characteristic of Chlamydia. Iodine, Giemsa, or immunofluorescence (IF) procedures are used for staining. Use of these stains must be timed to correspond with the occurrences in the developmental cycle. Iodine and Giemsa stains are used at 48-72 hours, which is near the end of the cycle when the inclusions are will formed and contain EBs, reticulate bodies, and glycogen. IF procedures can be used as early as 20 hours, because antigens at any stage of the cycle are detectible. Staining is not recommended after 72 hours since the number of inclusions drops rapidly as the cells rupture.

One or two of the cultures inoculated with each of the unknown specimens, the positive control, and the negative control is stained. (The staining of two cultures for each unknown may increase the chance of detecting inclusions.) The control cultures are examined to determine the sensitivity and specificity of the procedures used for isolation and staining. Negative control cultures are the base line to which all other cultures are compared. They should be free of inclusions and artifacts that may interfere with interpretation of results. Positive control cultures should have the expected number of inclusions. If the number of inclusions is much less than expected, false-negative results may occur.

Specimen cultures are considered Chlamydia-positive if one or more *typical* inclusion is observed. Cultures are considered negative if no inclusions are observed by 72 hours postinoculation. Negative cultures are passaged one time. If after the second passage, no inclusions are observed, the specimen is considered negative. The number of additional isolations that can be made by a third passage is negligible.

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Cultures with questionable or atypical inclusions are passaged, or the original material is reinoculated. Specimens that prove to be contaminated may be retreated with antibiotics and centrifuged lightly (200 x g, 5 minutes) and then reinoculated into cell culture.

Results of isolation attempts may be reported as follows:

Chlamydia Isolated: Positive Cultures

No Chlamydia Isolated: Negative cultures after 2 passages

Contaminated Specimen: Cultures contaminated after retreatment of the specimen.

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Passage of Negative Cultures

Cultures should be harvested at the peak of infectivity (48-72 hours). To enhance recovery of the organism, remove 1/2 (0.5 ml) of the culture medium from the vials and discard. Disperse the cell-bound Chlamydia organisms into the remaining medium by scraping the cells from the cover slip with a capillary pipette and mixing well, by adding a few glass beads to the culture and agitating with a mixer, or by sonicating the entire culture. If there is more than one culture for each specimen, they can be combined. If passage must be delayed, quickly freeze the culture and store it at −65° C or lower. At the time of inoculation, rapidly thaw the culture by agitating it in a 37° C water bath. The freeze-thaw process aids in rupture of the cells but reduces somewhat the infectivity of the suspension.

Passage of Cultures for Stock

The procedures used for passaging cultures are essentially the same as those used for isolation. To intensify the increase in the number of inclusions per passage, cultures can be concentrated by reducing the volume of medium into which the cells are dispersed, for example, 0.2–0.5 ml. This is done before the cells are ruptured by removing part of the culture medium or by removing all of the medium and replacing it with a smaller volume of a different medium. Another technique that may enhance Chlamydia growth is to leave the inoculum in contact with the cells for an additional 1-2 hours at 33° -35° C after centrifugation before removing it and adding the culture medium.

Preparation of Stock Cultures

Cultures are passaged until an inclusion count of at least 10^3/cover slip is reached. The organisms are dispersed into the medium as described above and the suspensions combined. Adding an equal volume of 4SP (0.4 M sucrose–0.02 M phosphate) solution to the pooled suspension may increase survival of the organisms. The suspension is dispensed in small volumes (0.5 – 1 ml) and frozen at −65° C or lower. Infectivity of the stock culture is determined by inoculating cell cultures with several dilutions (for example, undilute, 10^{-1}, 10^{-2}, and 10^{-3}) prepared from a frozen sample.

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Inclusion Counting

Since each inclusion body originates from one infectious particle, the number of inclusions present in a culture can be used as a measure of infectivity and as a basis for comparing experimental procedures. It is difficult to obtain a uniformly infected monolayer due to the growth characteristics of the organism and the techniques of cultivation. Infected cells often appear to be clustered rather than distributed evenly throughout the monolayer. As the number of inclusions increases, the more difficult it becomes to count all the inclusions present. For a cover slip which is moderately or heavily infected, it is more practical to count a sample of fields. Systematic field sampling, covering the entire cover slip, will give a good estimate of the total inclusions present. Based on the experience and data collected by the senior author and in consultation with CDC's statistical office, the following guidelines are suggested.

1. Determine the general degree of infectivity – low, moderate, or heavy – by scanning the cover slip.

   Low Infectivity – There are many fields that do not contain inclusions. In a high power field that contains inclusions, less than 10 inclusions are present.

   Moderate Infectivity – Most high power fields contain at least one inclusion, but a few fields with no inclusions are seen.

   Heavy Infectivity – Practically all fields have more than one inclusion.

2. Based on the degree of infectivity determined above, count each inclusion using the high dry objective.

   a. Low Infectivity – Examine and count the inclusions on the entire cover slip.

   b. Moderate and Heavy Infectivity – Make counts by systematic field sampling. Using the sampling patterns that follow, count 30 fields on moderately infected and 17 fields on heavily infected cover slips. Select each field without looking into the microscope. If a selected field is not a full field or cannot be completely counted due to monolayer damage, artifacts, and so forth, select another. Make at least two counts of each cover slip. Do each count in a different direction, for example, top to bottom and left to right. Determine the average number of inclusions per high power field. Diagrams of the fields are on the following page.

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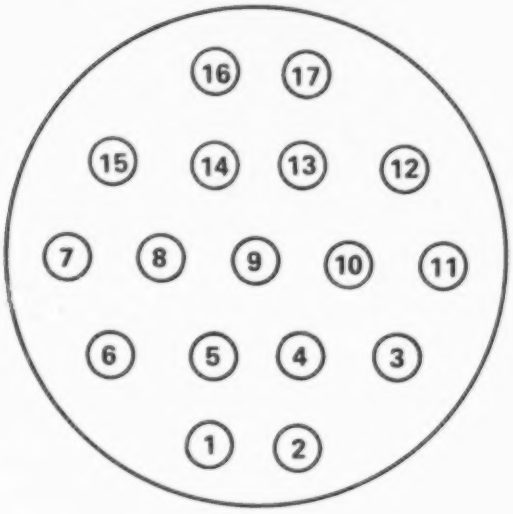
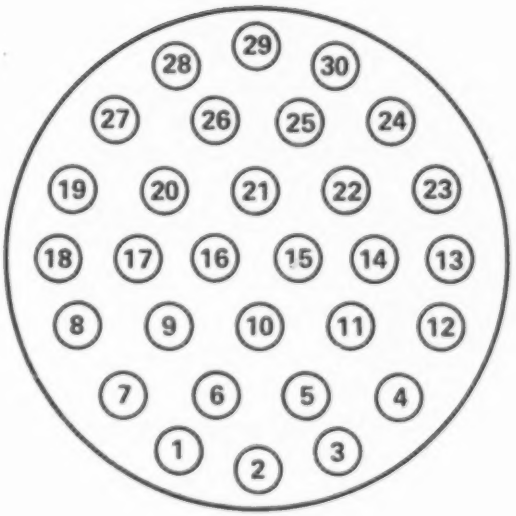


17 FIELDS



30 FIELDS

SECTION III

LABORATORY DIAGNOSIS OF *C. TRACHOMATIS* INFECTIONS

Part C – Serological Testing

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Introduction

Because of a number of problems, serological testing for the diagnosis of chlamydial infections is limited. No single test is totally satisfactory for all the Chlamydiae. Antibody response may be inadequate for measurement by certain tests. Paired sera may be difficult to obtain because infections may be inapparent or have a long incubation, which thwarts the collection of an acute serum. High-risk populations have high rates of background antibodies. In VD clinics, approximately 25% of the men and 60%-70% of the women from whom the organism is not isolated have Chlamydia antibodies.

Serum antibodies persist for extended periods. Detection of antibodies in a single serum indicates only previous exposure to the organism. A fourfold rise in antibody titer between an acute and a convalescent serum when accompanied by clinical symptomology supports the diagnosis of a chlamydial infection.

Antibodies in secretions (local antibody) are relatively short-lived; their presence appears to be related to an active/current infection. The presence of IgM antibody may be supportive of an active infection, but it is not always found. Occasionally, IgM can be found in cases without active disease. Detection of local or IgM antibody cannot be used exclusively for diagnosis; it is of value, however, for distinguishing recent from background antibody.

The level of antibody is related to the nature of the infection. The more invasive the organism, the higher the antibody titer. Highest titers are found in cases of active LGV; titers from cases of salpingitis are usually higher than those from cervicitis; titers from women are generally higher than those from men; and titers from culture-positive cases are higher than those from culture-negative cases. Those with a previous exposure to the organism will have higher reaction rates upon reinfection.

A number of serological tests have been described for use with Chlamydiae, but at present, only two tests are well established for *C. trachomatis* evaluations — the complement fixation (CF) test and the immunofluorescent (IF) test.

Complement Fixation

The CF test is group-specific — that is, the reacting antigen is common to all members of the Chlamydia genus — and cannot distinguish species or immunotypes. The test can be used to measure antibodies to psittacosis and LGV, but is relatively insensitive for

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measuring antibodies to oculogenital infections (*C. trachomatis* serotypes A-K). Significant titers (> 16) can only be demonstrated in no more than 50% of those infected with the oculogenital strains. With psittacosis and LGV, titers > 64 are common. Psittacosis is usually diagnosed by a fourfold rise in titer between an acute and a convalescent serum (paired sera). In chronic or relapsing cases, a high titer (> 64) of a single serum may be significant when accompanied by a clinical picture compatible with the disease. The diagnosis of LGV is usually based on clinical symptomology and the CF result of a single serum. A titer of 64 or greater is considered significant. It is rare to obtain an acute LGV serum because 2-3 weeks may elapse from the onset of the infection, which usually goes unnoticed, to the development of symptomology that makes the patient seek medical aid.

The procedure for CDC's standardized CF test can be found in the CDC publication, *A Guide to the Performance of the Standardized Diagnostic Complement Fixation Method and Adaptation to Micro Test.*

Micro-IF

The micro-IF is an indirect immunofluorescence test that uses minute volumes. It was developed in the early 1970's by Dr. Wang at the University of Washington to replace the cumbersome mouse toxicity prevention test for the typing of Chlamydia strains. In the test, antibodies react with the type-specific antigens found in the cell wall of the elementary bodies. The test is extremely sensitive for detecting, quantitating, and immunotyping antibody. It can be used to evaluate single serums for epidemiological surveys or to detect diagnostic titer rises in paired sera. The micro-IF test can also be used to identify and type isolates. Its ability to detect antibodies in culture-positive cases has been reported to range from 100% with LGV and adult inclusion conjunctivitis to 90% with urethritis. Antibodies in culture-negative cases can also be detected. In addition to serum, the micro-IF can be used to evaluate secretions such as those from the eye and genital tract. The test has been adopted by rickettsiologists as a sensitive method for rickettsial evaluations. It has also been used in studies with gonorrhea and Legionnaires' disease. An overview of the test is given in Figure 1. Figure 2 illustrates reaction patterns that occur with human serum.

Since its development, several modifications and/or variations in the micro-IF test have increased its versatility and practicality. Most

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notable are changes in the type and usage of the antigens. Antigens can be prepared from infected cell culture as well as from yolk sac, as was done originally. The antigens can be treated with formalin which not only renders them noninfectious but increases their stability, permitting storage for extended periods (possibly years) at 4° C. Formalin treatment may also increase specificity. Originally, separate antigen dots were used for each immunotype, but, for most purposes, pooling of closely related immunotypes has been found to be more practical. A suggested set of pools is as follows: C/J, E/D, F/G, and K/L_3. A, B, H, and I are more antigenically distinct and are not pooled. A L_1/L_2 pool can be included in the battery, but it is usually not needed for most evaluations. Some investigators use only antigens to the immunotypes prevalent in their specimen source population; others use only a single, broadly reactive antigen such as L_1 or L_2. *No single antigen, however, will detect antibody to all the immunotypes.*

The test can be further simplified by reducing the number of serum dilutions. This can be accomplished by either increasing the serum dilution factor, for example, fourfold instead of twofold dilutions or by increasing the initial dilution, for example, 1:8.

Versatility of the test can be increased by the use of monospecific conjugates – IgM, IgG, IgA, or secretory piece.

When typing isolates, instead of preparing the test antigen from the isolate as is often done with other agents, it is more practical to inoculate mice with the isolate and test the mouse serum against known antigens.

Modifications in the test may affect the sensitivity or specificity; therefore, any modification should be carefully selected so that the needs of the investigator are met without sacrificing the reliability of the results.

ELISA

The ELISA is a group specific test that appears to be more sensitive than the CF test for detecting Chlamydial antibody. Data on the use of ELISA is limited to the evaluation of sera from psittacosis and LGV infections. ELISA has not, as yet, been fully evaluated for use with oculogenital infections.

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Figure 1. Overview of the Steps and Reactions in the Micro-IF

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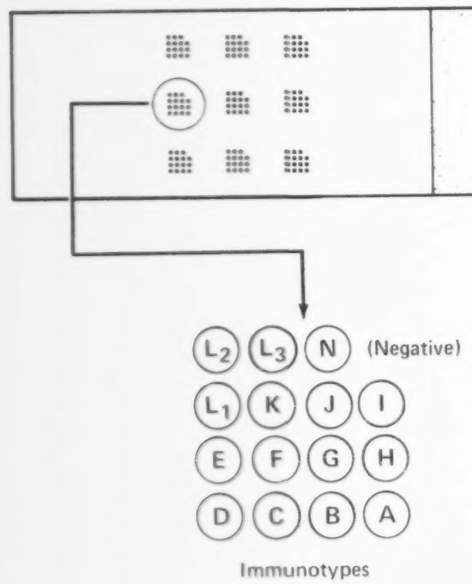
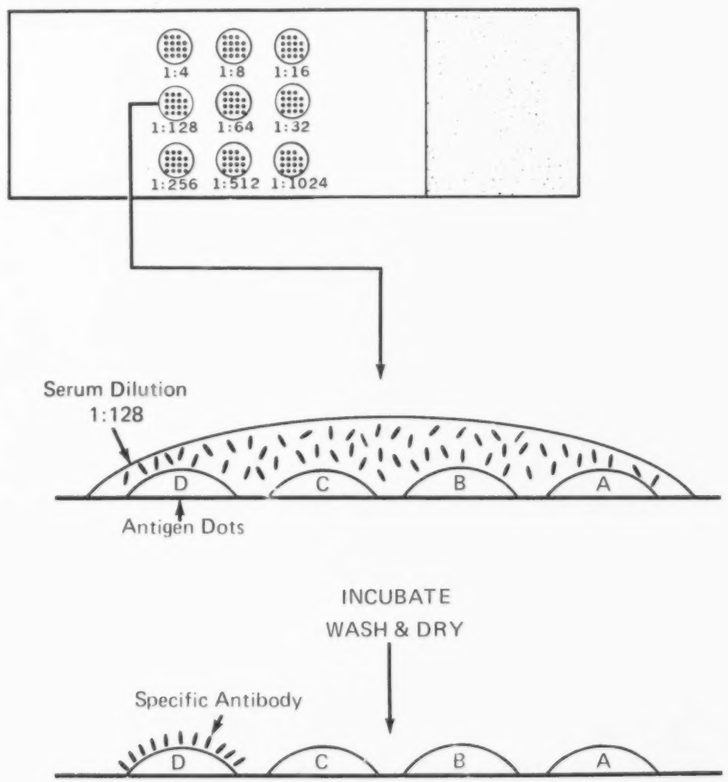
Step 1: Preparation of the Slides

Small dots of antigen for each immunotype are placed in groups with a specific pattern on a microscope slide. Slides are air-dried and fixed in acetone.



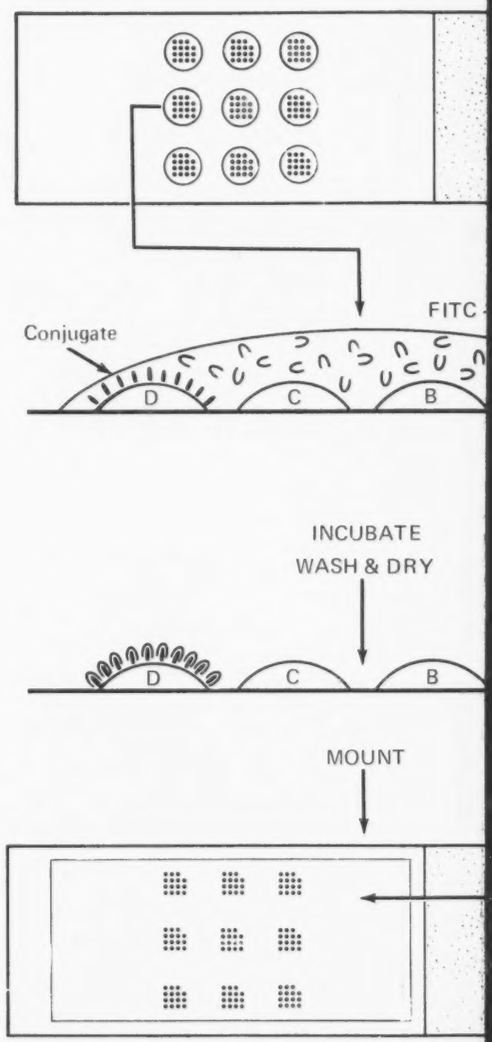
Step 2: Addition of Serum Dilutions

The serum (or secretion) is diluted. Each dilution is added to one group of antigen dots with a bacteriological loop or micropipettor (<10μl). Slides are incubated, washed, and dried. Specific antibody adheres to its homologous antigen.



Step 3: Addition of the Conjugate

A working dilution of an FITC antispecies conjug
group of dots. Slides are incubated, washed, and
reacts with the antibodies adhering to the antigen.



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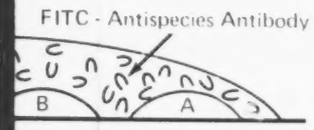
es conjugate is placed on each
hed, and dried. The conjugate
antigen.



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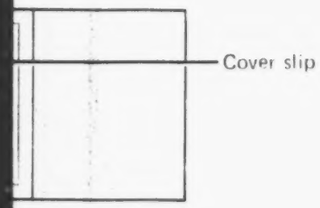

Step 4: Examination of Stained Slides

Slides are viewed with a fluorescent microscope (the image is reversed). Each antigen dot is examined and evaluated for the prescence of specific fluorescence. The result of each antigen dot is recorded.

Step 5: Interpretation

The results to the right are examined. The reaction of each dilution of serum with each antigen is noted and the serum dilution end point determined. Since Chlamydia immunotypes are highly interrelated, positive reactions frequently occur with more than one type of antigen. As the serum is diluted, the range of reactivity narrows as the reaction becomes more type specific. Certain immunotypes such as E/D, G/F, and C/J are so closely related that they react almost identically and often cannot readily be distinguished.

Results

1:4

A	B	C	D
–	+	–	+
H	G	F	E
–	+	+	+
I	J	K	L1
–	–	+	+
	N	L3	L2
	–	+	+

1:8

A	B	C	D
–	+	–	+
H	G	F	E
–	+	+	+
I	J	K	L1
–	–	+	+
	N	L3	L2
	–	+	+

1:16

A	B	C	D
–	+	–	+
H	G	F	E
–	+	+	+
I	J	K	L1
–	–	±	+
	N	L3	L2
	–	+	+

1:128

A	B	C	D
–	–	–	+
H	G	F	E
–	–	–	+
I	J	K	L1
–	–	–	±
	N	L3	L2
	–	–	±

1:64

A	B	C	D
–	–	–	+
H	G	F	E
–	–	–	+
I	J	K	L1
–	–	–	+
	N	L3	L2
	–	±	+

1:32

A	B	C	D
–	+	–	+
H	G	F	E
–	–	±	+
I	J	K	L1
–	–	–	+
	N	L3	L2
	–	+	+

1:256

A	B	C	D
–	–	–	±
H	G	F	E
–	–	–	+
I	J	K	L1
–	–	–	–
	N	L3	L2
	–	–	–

1:512

A	B	C	D
–	–	–	–
H	G	F	E
–	–	–	–
I	J	K	L1
–	–	–	–
	N	L3	L2
	–	–	–

1:1024

A	B	C	D
–	–	–	–
H	G	F	E
–	–	–	–
I	J	K	L1
–	–	–	–
	N	L3	L2
	–	–	–

Serum Dilution End Points

A	B	C	D	E	F	G	H	I	J	K	L1	L2	L3
*	32	–	128	256	16	16	–	–	–	8	64	64	32

* = 1:4
∴ Immunotype: E (D)
Titer: 256

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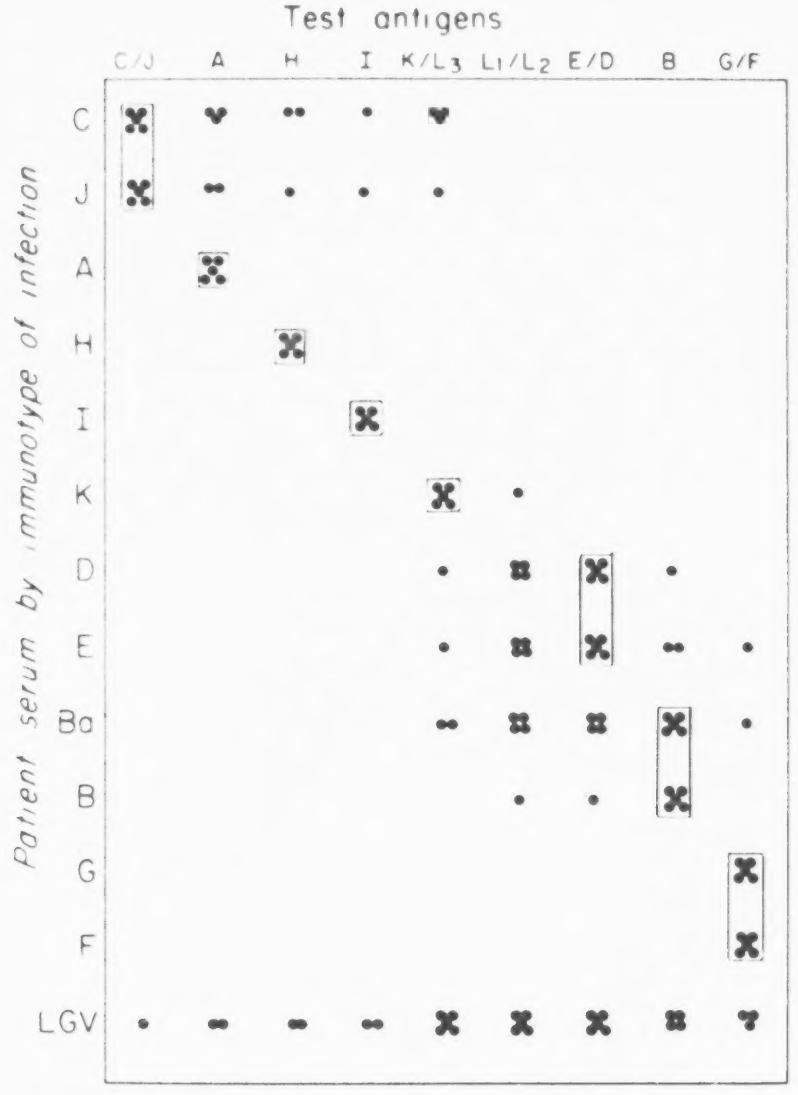
Figure 2. Reaction Patterns Expected With Human Sera in the Micro-IF Test



Schematic expression of reaction patterns in the micro-IF test of patient sera derived from infection with different immunotypes of *C. trachomatis.* Homologous reaction is expressed by five dots. Difference in number of dots shows extent of cross-reaction in terms of titer difference from homologous titer in test with twofold dilutions of serum.

Source: Wang S-P, Grayston JT, Kuo C-C, Alexander ER, Holmes KK: Serodiagnosis of *Chlamydia trachomatis* Infection with the Micro-Immunofluorescence Test in Hodson D, Holmes KK (eds): Nongonococcal Urethritis and Related Infections. Washington, D.C., American Society for Microbiology, 1977, p. 240. Reprinted with permission.

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Procedure For The Micro-IF

Part I — Preparation of Substrate Slides

Materials

Microscope slides, precleaned

Template

Antigens: Positive (Immunotype Pools G/F, K, H, I, E/D, B, A, and C/J)

Negative Control (Host tissue)

Forceps, small blunt

Lock washers, 3/8-inch

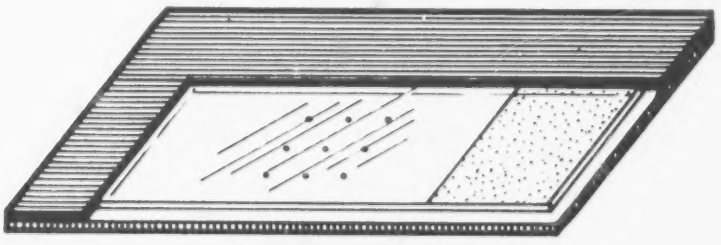
Pen points, "Hunt" size 104 or equivalent

Vortex mixer

Acetone

Procedure

1. Dip precleaned slides in acetone and wipe dry. Repeat. Slides may be precleaned by soaking overnight in a dicromate cleaning solution and rinsing thoroughly in distilled water.
2. Place a clean slide over the template as illustrated below with the frosted end to the right.



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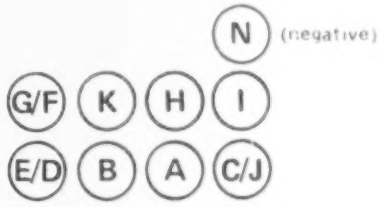
3. Determine the antigen placement pattern to be used. A commonly used pattern is illustrated below:



4. Grasp a pen point with forceps and lock it in place with a washer as illustrated.

5. With a vortex or similar mixer, mix the first antigen (C/J) to be placed on the slide.

   NOTE: Antigens, if not formalinized, are stored frozen at -65° C in small amounts (0.2ml). For use, they are quickly thawed at 37° C. Thawed antigens are stored at 4° C and may be used for 4-5 days, after which time they are discarded.

6. Dip the pen point into the antigen. Pretest the delivery of the antigen on a clean slide until uniform dots, which do not run, are delivered.

7. Place a dot of the first antigen (C/J) on the slide over each of the dots in the template. A single filling of the pen is usually sufficient for several slides.

8. Remove the slide containing the antigen dots from the template, set aside. Place another slide over the template. Repeat steps 5-7, still using the first antigen. Prepare as many slides as will be needed.

9. After all dots of the first antigen have been placed on the slides, release the pen point from the forceps and drop it into a small beaker of alcohol/formalin for decontamination. Decontaminated pen points are cleaned by scrubbing with a small brush (toothbrush) and quickly dried to prevent rusting.

10. Using the procedures in steps 5-9 and the first dot as a guide, place the remaining antigens, one at a time, on the slides according to the predetermined pattern. Note that in the

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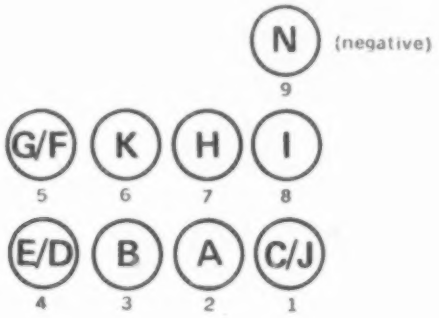
example, the second antigen (A) is placed to the *left* of the first dot and the second row of antigen dots is *above* the first row.



11. Completely air-dry the slides for a minimum of 30 minutes after adding the last antigen dot. Dots must be completely dry before fixation.

12. Fix dried slides in acetone for 10-15 minutes at room temperature. (Absolute methanol can also be used as a fixative.)

13. Proceed with the addition to the sera to the slides.
NOTE: Fixed slides can be stored overnight at 4° C or for longer periods if frozen.

Part II – Addition of Sera or Secretions and Conjugate

Materials

Substrate slides, fixed

Sera: Known Positive
Known Negative
Unknown Test Specimens

Phosphate Buffered Saline (PBS), pH 7.2

Conjugate(s), FITC-labeled antispecies, working dilution

Bacteriological loop or micropipettor with a delivery of approximately 10 microliters or less

Mounting medium

Cover slips

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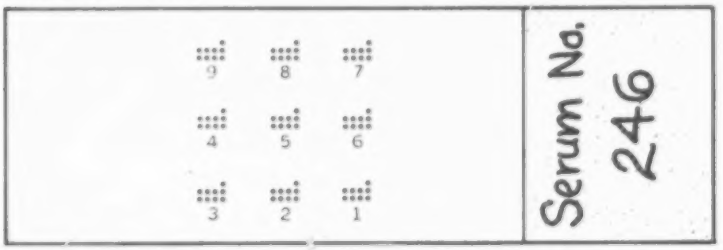
Procedure

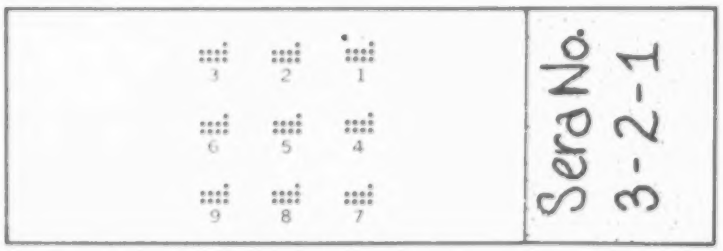
1. Dilute each serum in PBS in 0.5-ml volumes. For serum, the starting dilution is usually 1:8; for secretions, 1:10. Either 2-fold or 4-fold dilutions are prepared.
2. With the frosted end to the right, label each slide to correspond to the sera being tested.
3. Starting with the highest dilution of serum, add one loopful (or one drop from a micropipettor) of each dilution to groups of antigen dots in the numbered sequence (1-9) illustrated below starting with 1. The frosted end of the slide should be on the right. Between dilutions, blot the loop on a clean spot on a piece of absorbent paper.

One Serum/Slide



1=1:2,048, 2=1:1,024, 3=1:512 . . . . . .

Three Sera/Slide



Serum #1 – 1=1:128, 2=1:32, 3=1:8

Serum #2 – 4=1:128, 5=1:32, 6=1:8

Serum #3 – 7=1:128, 8=1:32, 9=1:8

4. Place slides in a moist chamber at 37° C and incubate for 30 minutes.

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5. Rinse slides in 4 changes of PBS and 3 changes of distilled water. Briefly blot the edge of the slides on absorbent toweling between each change of solution.

6. Stand slides on end and allow to air-dry.

7. Add a drop of the working dilution of conjugate to each group of antigen dots with a bacteriological loop or a micropipettor.
   NOTE: Conjugates used for Chlamydia testing are usually prepared from goat serum and labeled with fluorescein isothiocyanate (FITC). Antimouse, antihuman, and monospecific IgM, IgG, IgA, and secretory piece conjugates are commercially available. Conjugates are stored according to the manufacturer's recommendations. Before use in a diagnostic test, the conjugate is titered. The optimal, working dilution is determined as the highest dilution that gives intense *specific* staining but negligible background staining. Working dilutions of conjugate are prepared in PBS and, if necessary, clarified by passing through a membrane filter (0.45μm) or by high-speed centrifugation, for example, 1,500 x g, for 30 minutes at 4° C.

8. Place slides in a moist chamber at 37° C and incubate for 30 minutes.

9. Rinse slides as before in 4 changes of PBS and 3 changes of distilled water, blotting between each solution.

10. Slides may be air-dried before mounting.

11. Mount slides with cover slips and a glycerine/buffered saline mounting medium, pH 7.2 (available commercially).
    If not examined immediately, store stained slides in the dark at 4° C. Slides should be examined within 24-48 hours after staining.

Part III — Evaluation of Stained Slides

Materials

Stained slide preparations

Fluorescence microscope with an FITC excitor filter (KP-500) or a BG-12 excitor filter

Immersion oil (low fluorescence)

Record sheets

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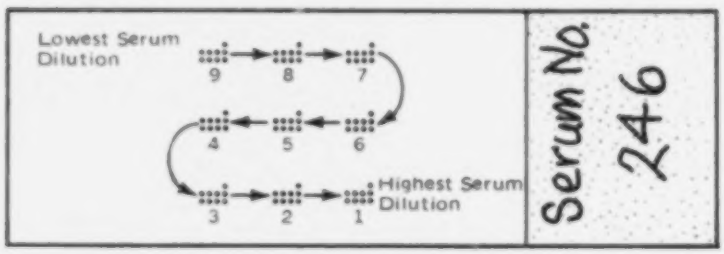
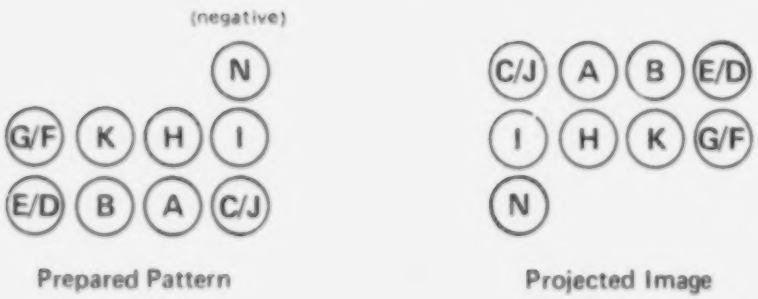
Procedure

1. Place a stained slide under the microscope with the frosted end to the right and the group of antigen dots in the upper left corner (9) under the low-power objective. As the slide is moved from right to left, the groups of antigen dots will read left to right; thus, the lowest serum dilution is read first. The microscope reverses the image so the pattern of antigen dots is reversed, as illustrated below.*





*NOTE: The image will appear as indicated above using a conventional microscope in which the eyepieces are *behind* the stage. the image will be different, however, if a microscope with front-mount (eyepieces and stage on the same side) is used.

2. With the low-power objective (10X-16X), locate the C/J antigen dot (upper left corner of project of image) in antigen group No. 9 (upper left corner).

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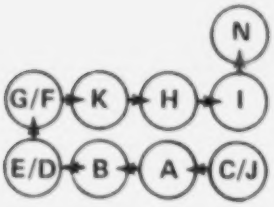
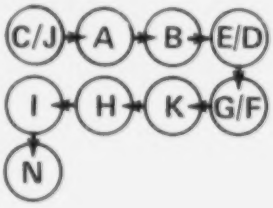
3. Switch to the high-power objective (40X-100X), focus, and begin reading the dots in the first antigen group (the lowest serum dilution) following the pattern below.



Prepared Pattern

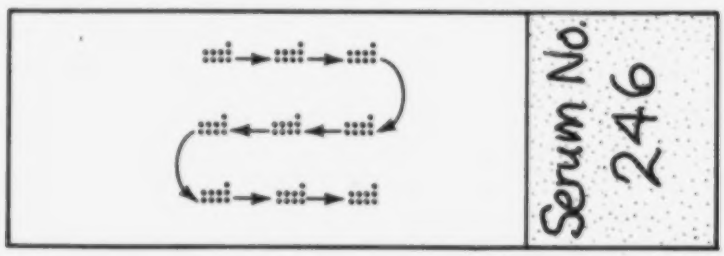


Projected Image

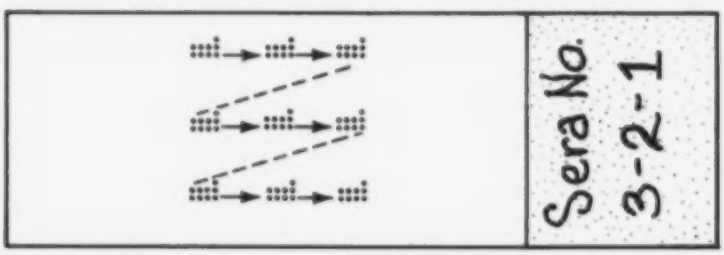
4. Evaluate each dot in the first antigen group and record the results. The elementary bodies are dispersed throughout the antigen dot and will fluoresce brightly if the serum contains specific antibodies. Dots are evaluated by the homogenicity and intensity of the fluorescence, but not by the amount of antigen that fluoresces. Fluorescence that appears only around the edge of the dot or in isolated areas is considered nonspecific. Specific fluorescence will be bright and associated with particulate material (the elementary bodies) that is evenly distributed throughout the dot. The intensity of the specific fluorescence is graded as high positive (++), positive (+), and minimal (±).

5. Evaluate and record the results of each dot in the remainder of the antigen groups in the appropriate sequence illustrated below.

One Serum/Slide



Three Sera/Slide



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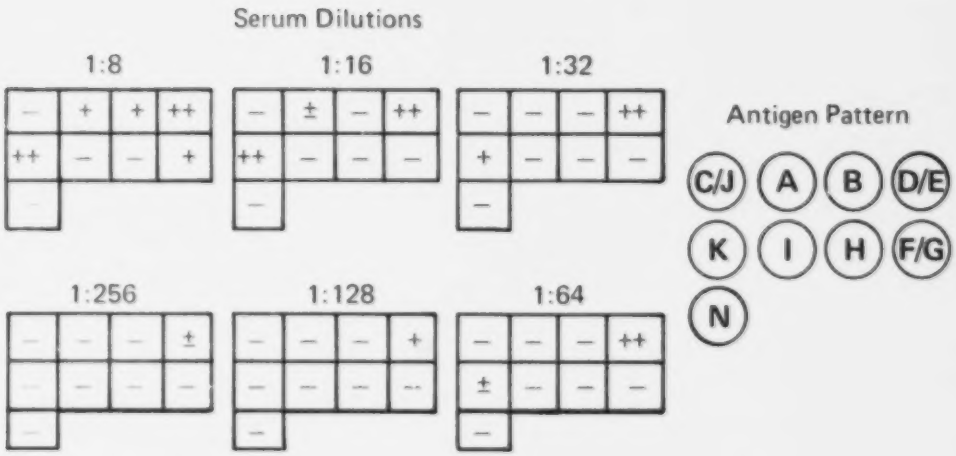
6. First examine the results of the positive and negative control sera. The negative control serum should not react with any of the antigen dots. The positive serum should react as expected from previous testing. If the control sera are reacting properly, proceed with the interpretation of the unknown test sera results.

7. Examine the results of each test serum and determine the dilution end point for each antigen. The end point is the highest dilution with positive (+) fluorescence. Fluorescence in the negative control antigen negates other reactions at that dilution. An example showing the determination of the serum dilution end points follows.

Results of Serum No. 246



Serum Dilution End Points

Immunotypes:	C/J	A	B	D/E	F/G	H	I	K	(N)
Serum No. 246	<8	8	8	128	8	<8	<8	32	<8

Thus the titer of the serum is 128 and the predominate antibodies are to immunotype D/E.

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Immunotyping of Isolates

See Figure 3 for an outline of the procedure.

Part I – Preparation of Immunizing Antigen

Materials

Original Isolate in Cell Culture (McCoy)

Cell Cultures and Media (Same system as used for isolation)

SPG

Staining Reagents

Procedure

1. Of the cultures that were inoculated originally, two remain after one is stained. Freeze one of these at −65° C or lower as a reserve.
2. Remove the medium from the remaining culture. Add 0.5 ml SPG.
3. Rupture the cells to suspend the elementary bodies.
4. Inoculate 4 new cultures, each with 0.1 ml of the suspension. Follow the procedure previously described on p 51.
5. After 48 – 72 hours' incubation, stain 1 of the cultures by one of the staining methods previously given, pp 57-66.
6. Using a microscope, examine the stained preparation with a high power objective.
7. Count the inclusions in at least 17 high-power fields. Determine the average count and record the number.
8. Passage the remaining 3 cultures using the procedures described in steps 2 – 7. The cells from each culture are suspended in 0.2 ml volumes which are combined as inoculum for the next passage.
9. Continue passing the material until the inclusion count reaches 40 – 50 per high-power field (HPF). Four cultures are inoculated with each passage.

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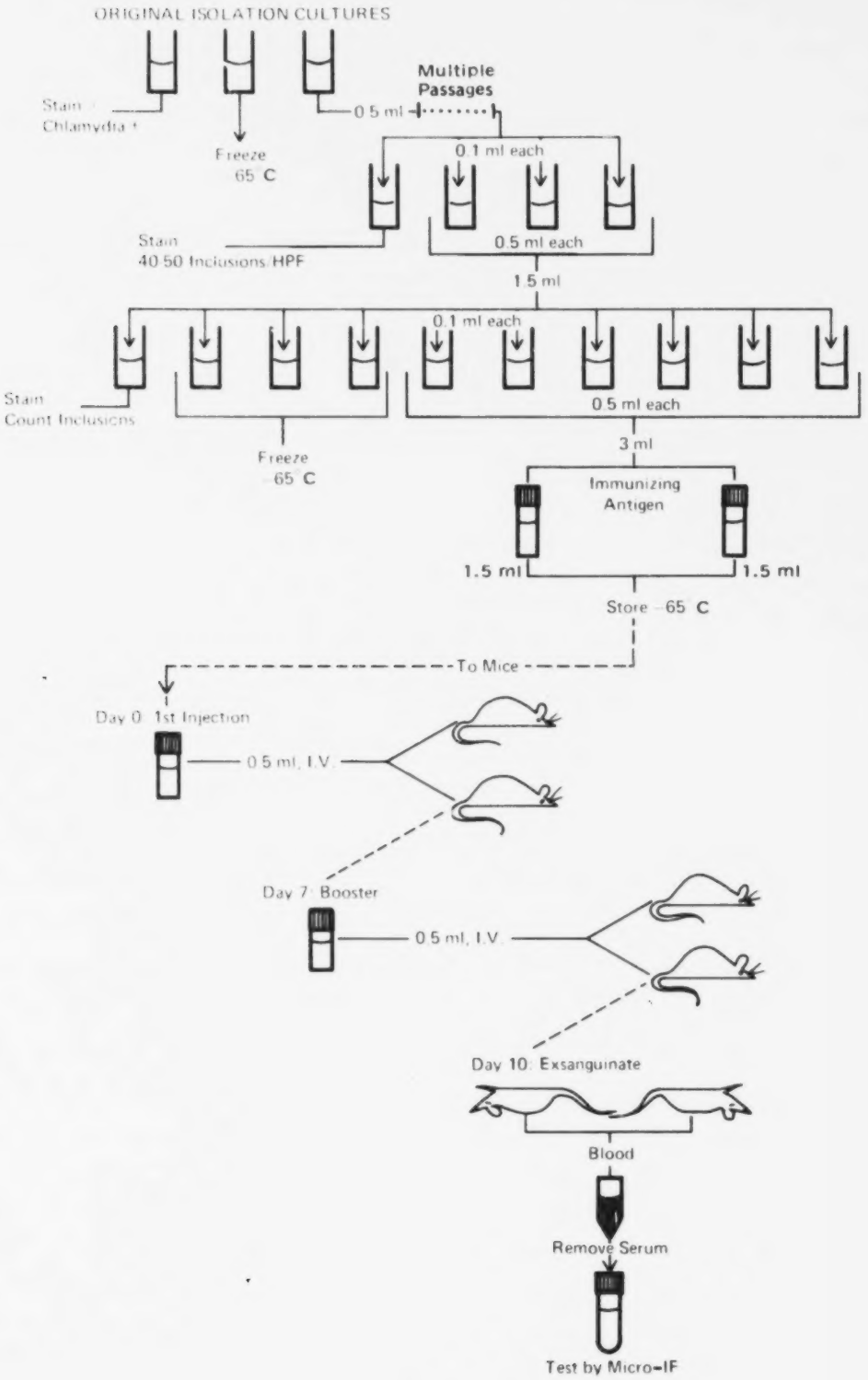
Figure 3. Outline of the Procedure for Immunotyping an Isolate



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10. When sufficient inclusions are present (40-50/HPF), suspend the cells of the remaining 3 cultures in 0.5 ml volumes. Combine and use as inoculum for 10 new cultures (0.1 ml each).

11. At 48 – 72 hours, use 1 of the 10 cultures for staining and evaluation; freeze 3 cultures as a reserve; and prepare immunizing antigen from the remaining 6 cultures as follows:

    a. Remove the cell culture medium and add 0.5 ml SPG to each vial. Rupture the cells and suspend the elementary bodies as before.

    b. Combine the contents of the 6 cultures (total volume = 3 ml). Mix well and divide into 2 aliquots of 1.5 ml each. This is the immunizing antigen. Store frozen at −65° C or lower.

Caution: Be extremely careful so as not to mix material from different isolates.

Part II – Immunization of Mice

Materials

Mice, 4-5 weeks old, 2/isolate

Immunizing Antigen, 2-1.5 ml of each isolate

Anesthetic

Procedure

1. Inject 0.5 ml of the immunizing antigen into the tail vein (i.v.) of a 4-5-week-old mouse. Inoculate 2 mice for each isolate.

2. Place the 2 mice inoculated with the same isolate together in a cage that has been clearly labeled.

3. On day 7, give each mouse a second injection – 0.5 ml, i.v.

4. On day 10, exsanguinate each mouse as follows:

    a. Anesthetize the mouse with ether, CO_2, or a drug. If CO_2 is used, mice must be watched very closely since their tolerance to CO_2 is very low and they die easily. A drug, pentobarbitol sodium, can be used by injecting the mouse intraperitoneally

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(i.p.) with 0.1 ml of a solution that gives a final dosage of 100 μg/mouse. Other drugs such as ketamine and Innovar are also suitable.

b. Exsanguinate the anesthetized mouse by one of the following methods:

Cardiac puncture – Remove the blood from the heart with a needle and syringe. Transfer the blood to a conical centrifuge tube.

Auxillary slice – Secure the mouse on its back. Cut skin and tissue to expose the brachial plexus. Sever the artery and allow the blood to flow into the pocket that is formed. With a capillary pipette, slowly withdraw the blood from the pocket and transfer it to a conical centrifuge tube.

Jugular slice – Hold the mouse over a conical centrifuge tube. Sever the jugular vein under the jawbone with a sharp razor blade or scalpel. Allow the blood to drip into the centrifuge tube.

c. Immediately after bleeding, euthanize the mouse with an overdose of anesthetic.

Combine the blood of the two mice in a conical centrifuge tube. Approximately 0.5-1 ml of blood can be obtained from each mouse.

5. Obtain blood from uninoculated mice from the same colony as a control.

6. Allow blood to clot. Ring the clot and centrifuge at 650 x g for 30 minutes.

7. Remove the serum and test it by the micro-IF test.

Modification: In cases of extreme urgency, a small sample of blood can be taken on day 4 by clipping off a small piece of the tail and collecting the blood in a centrifuge tube.

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Part III – Testing by Micro-IF

Materials

Substrate slides

Mouse Sera: Normal (Uninoculated)
Test (Isolate Inoculated)

PBS

Conjugate, FITC antimouse

Mounting medium

Procedure

1. Starting at 1:8, make 8, 2-fold dilutions of each serum in PBS (1:8 – 1:1024).

2. Perform the micro-IF as previously described (p 80) using anti-mouse conjugate. Antigen pools are to be used for initial testing. If the individual immunotype cannot be identified, the serum is retested by using antigens to the individual immunotypes. See Figure 4.

3. If the test results are negative, the entire procedure is repeated. New immunizing antigen is prepared from the 3 cultures that were frozen in step I-11.

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FIGURE 4. Testing of Mouse Sera to Determine Immunotype of Isolate: Examples of Micro-IF Results

Initial Testing: Antigen Pools

Mouse Serum	Antigens C/J	A	B	E/D	G/F	K	H	I	Identity Immunotype
#1	<8	8	8	128	8	32	<8	<8	E/D
#2	<8	<8	<8	8	128	8	<8	<8	G/F
#3	32	8	<8	<8	<8	<8	<8	8	C/J
#4	<8	8	<8	8	8	8	<8	128	I

Second Testing: Individual Antigens

Mouse Serum	Antigens C	J	E	D	G	F	K	I	Identity Immunotype
#1	<8	<8	32	64	<8	8	32	<8	D
#2	<8	<8	<8	<8	32	128	<8	<8	F
#3	8	32	<8	<8	<8	<8	<8	<8	J
#4	<8	<8	<8	<8	<8	<8	8	128	I

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Preparation of Antigen for the Micro-IF

Materials

Stock Culture (Prototype strain of an immunotype)

Cell Culture Monolayers (McCoy) contained in:

1-dram shell vials with 12 mm round cover slips,

75-cm^2, and 150-cm^2 cell culture flasks

Culture medium containing Cycloheximide

SPG

PBS, pH 7.2

Glass Beads, 4 mm

2% Normal Yolk Sac in PBS, pH 7.2 containing 0.02% Formalin

Procedure

1. Pass the stock culture as described in the procedure for the preparation of the immunizing antigen (p 88) until the inclusion count reaches 40-60/HPF. A minimum of 20 1-dram vial cultures is needed.
2. Remove the culture medium and add 0.25 ml of SPG to each of the 20 cultures. Rupture the cells.
3. Combine the suspensions of the 20 cultures (approximately 5 ml).
4. Remove the growth medium from cell culture monolayers in two 1-dram vials and one 75-cm^2 flask.
5. Using the 5-ml suspension prepared in step 3 as inoculum, inoculate the 2 vials with 0.1 ml each and the 75-cm^2 culture with the remainder. Rock the cultures to spread the inoculum.
6. Place the cultures at 35° C for 3-4 hours. Periodically, rock the cultures.
7. Add 30 ml culture medium containing cycloheximide to the 75-cm^2 flask and 1 ml to each of the vials. Incubate the cultures for 48 hours at 35° C.

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8. After 48 hours incubation, remove the culture medium from the cells. Add 10 ml SPG to the 75-cm² culture and rupture the cells. Stain the cells on the cover slips in the vials. Microscopically examine the stained cells and determine the per cent of cells infected.

9. If less than 80% of the cells are infected, pass the harvest (10 ml) from the 75-cm² flask. Inoculate two new 75-cm² flasks and two 1-dram vials with the 10 ml prepared in step 8.

10. Continue to pass the material in 75-cm² flasks until 80% to 100% of the cells are infected.

11. When 80% to 100% of the cells are infected, use the 10 ml prepared from a 75-cm² flask to inoculate one 150-cm² flask and two vial cultures. Use the procedures described in steps 4-7. Add 50 ml of medium containing cycloheximide to the 150-cm² culture.

12. Incubate the cultures at 35° C for 48 hours.

13. At 48 hours, stain and examine the cells from the vial cultures to ascertain that 80% to 100% of the cells are infected. If the degree of infectivity is sufficient, process the 150-cm² culture for antigen. If the infectivity is not sufficient, pass the material.

14. Prepare the antigen as follows:

    a. If the infection has not caused the cells to rupture, decant the medium and proceed to step 2. If the cells have ruptured, the medium must also be processed.

    b. Add 5 ml of PBS and a few glass beads to the 150-cm² flask.

    c. Rock the flask until the cells are detached and suspended in the medium.

    d. Decant the cell suspension into a 30-ml centrifuge tube.

    e. Rinse the culture flask with an additional 10 ml of PBS. Add the fluid to the cell suspension in the centrifuge tube.

    f. Sonicate the cell suspension. (With a probe-type sonicator, 20 seconds is usually sufficient.)

    g. Centrifuge the suspension at 500 x g for 20 minutes at 4° C.

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h. Remove the supernantant fluid and centrifuge it at 30,000 x g for 60 minutes at 4° C.

i. Remove and discard the supernatant fluid.

j. Resuspend the pellet in 2% normal yolk sac in PBS, pH 7.2 containing 0.02% Formalin. Use 2 ml for each 150-cm² culture.

k. Dispense the antigen in small amounts (0.2 ml) and freeze at −65° or lower.

Alternate Procedure: Resuspend the pellet in PBS containing Formalin at a final concentration of 0.02%. Use 2 ml for each 150-cm² flask. Sonicate the suspension and store it at 4° C. For use, combine the amount of antigen needed with a suspension of normal yolk sac (prepared in PBS-0.02% Formalin) to give a final yolk sac concentration of 2%. The antigen diluted in yolk sac can only be stored at 4° C for a few weeks.

15. Prepare individual antigens for each immunotype. Antigen pools are prepared by combining equal volumes of individual antigens of two immunotypes, for example, equal parts D and E.

SECTION IV
REFERENCES AND SUGGESTED READING

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SECTION V

APPENDIX

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2 SP (0.2 M Sucrose – 0.02 M Phosphate)

Sucrose	68.46 g
K_2HPO_4	2.01 g
KH_2PO_4	1.01 g

Dissolve each ingredient separately in distilled water. Combine the three solutions. Bring the total volume up to 1,000 ml with distilled water.

Autoclave 20 minutes. Allow the solution to cool.

Add antibiotics.

Dispense in convenient volumes (0.5 – 2.0 ml).

Store at 4° C (short term) or −20° C (long term).

4 SP (0.4 M Sucrose – 0.02 M Phosphate)

Sucrose	136.92 g
Na_2HPO_4	2.268 g

Dissolve sucrose in 600 ml distilled water and Na_2HPO_4 in 200 ml. Combine the two solutions and add distilled water to a final volume of 1,000 ml. Adjust pH to 7.0 – 7.1 with 1 N HCl

Autoclave 20 minutes. Cool and dispense in convenient volumes.

Store at 4° C.

SPG (Sucrose Phosphate Glutamate)

Sucrose	75.0 g
KH_2PO_4	0.52 g
K_2HPO_4	1.22 g
Glutamic acid	0.72 g

Dissolve ingredients in distilled water to a total volume of 1,000 ml.

Adjust pH to 7.4 – 7.6. Sterilize by membrane (0.2μm) filtration. Add antibiotics. Dispense in convenient volumes. Store at 4 °C.

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Antibiotics

Antibiotic	Final Concentration
Gentamicin	10 μg/ml
Nystatin (Mycostatin)	25 units/ml
Streptomycin	50 μg/ml
Vancomycin	100 μg/ml
Neomycin	100 μg/ml
Amphotericin B (Fungizone)	2 μg/ml

The table above lists some of the antibiotics that can be incorporated into transport and cell culture medium. Antibiotics are used in combinations such as the following:

Gentamicin – Nystatin

Streptomycin – Vancomycin – Nystatin

Streptomycin – Neomycin – Nystatin

Amphotericin B can be toxic to cell culture; it is important that its concentration not exceed the 2 μg/ml.

When adding antibiotics to medium, the shelf life of each antibiotic must be taken into account. The time of addition and the conditions of medium storage should be such that the maximal effectiveness of the antibiotic is retained.

0.01 M PBS (Phosphate Buffered Saline), pH 7.2

Stock Solutions:

0.1 M Na_2HPO_4 (anhydrous)	14.2 g/liter
0.1 M NaH_2PO_4	26.8 g/liter
8.5% NaCl	85.0 g/liter

Prepare each stock solution separately by dissolving the ingredient in distilled water and then adding water to a final volume of 1,000.

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Working Solution, pH 7.2:

0.1 M Na_2HPO_4	28.8 ml
0.1 M NaH_2PO_4	72.0 ml
8.5% NaCl	100.0 ml
Distilled water	800.0 ml

PBS can be sterilized by autoclaving for 20 minutes.

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Eagle's CDC's Modified

Component	mg/liter
NaCl	6,800.0
KCl	400.0
$MgCl_2 \cdot 6H_2O$	200.0
$CaCl_2$	200.0
$NaH_2PO_4 \cdot H_2O$	133.0
Biotin	1.0
Choline	1.0
Folic Acid	1.0
Inositol	2.0
Nicotinamide	1.0
Pantothenic acid	1.0
Pyridoxal HCl	1.0
Riboflavin	0.1
Thiamine HCl	1.0
L-Arginine	127.0
L-Cystine	24.0
L-Glutamine	300.0
L-Histidine $HCl \cdot H_2O$	42.0
L-Isoleucine	52.0
L-Leucine	52.0
L-Lycine HCl	72.0
L-Methionine	15.0
L-Phenylalanine	32.0
L-Threonine	48.0
L-Tryptophane	10.0
L-Tryrosine	36.0
L-Valine	46.0
Glucose	5,000.0
$NaHCO_3$	2,000.0
Phenol red	20.0

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Serum

Sterile serum is available commercially. Fetal bovine serum is usually preferred for cell culture and Chlamydia growth. At present, however, it is in short supply, necessitating the use of other kinds of serum such as newborn bovine.

Regardless of the kind, all serum should be pretested to ascertain that it is supportive of cell culture and Chlamydia growth; that it does not contain inhibitors, antibodies, or toxic substances; and that it is free of contaminants – mycoplasmas, bacterias, and fungi.

Store fresh serum frozen at −20° C. Before use, inactivate serum by heating at 56° C for 30 minutes. Store inactivated serum at 4° C. Asceptically add inactivated serum to sterile medium to the desired concentration.

HEPES Buffer

1 M Stock Solution (50X concentrate):

HEPES	23.83 g
Double distilled water	100.0 ml

Equilibrate overnight in a glass container. Adjust the pH to 7.5 with 1 N NaOH. Sterilize by membrane (0.2μm) filtration. Store at room temperature.

Dilute the stock solution 1:50 in medium to give a final concentration of 0.02 M: 20 ml of 1 M stock is required for 1 liter of medium.

Composition of Media

Cell Culture Growth Medium:

Eagles's CDC's Modified containing

Fetal calf serum	10%
Gentamicin	10 μg/ml
Nystatin	25 units/ml

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Medium for Chlamydia Growth:

Eagle's CDC's Modified containing

Fetal calf serum	10%
HEPES buffer	0.02 M
Gentamicin	10 μg/ml
Nystatin	25 units/ml

Hanks' BSS (Balanced Salt Solution)

Component	mg/liter
NaCl	8,000.0
KCl	400.0
$MgSO_4 \cdot 7H_2O$	200.0
$CaCl_2 \cdot H_2O$	185.0
Na_2HPO_4	46.0
KH_2HPO_4	60.0
Glucose	1,000.0
$NaHCO_3$	350.0
Phenol Red	20.0

PBS (Phosphate Buffered Saline), Mg^{++} and Ca^{++} Free

NaCl	8.0 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g
Distilled water q.s. to	1,000.0 ml

Sterilize by autoclaving at 121° C for 20 minutes

Trypsin

Crude trypsin preparations are usually more effective than preparations from purified trypsin. Crude trypsin can vary in activity from lot to lot; thus each lot must be evaluated.

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Stock Solution (10%):

Crude Trypsin (1:300)	10 g
PBS, Mg^{++} and Ca^{++} free	100 ml

Mix by stirring for 10 minutes at 4° C. Centrifuge the solution for 1 hour at 10,000 x g. Sterilize by filtration. (A Hormann D9T filter is used at CDC.) Aliquot and freeze at −20° C.

Working Solution:

Dilute the stock solution in PBS, Mg^{++} and Ca^{++} free, to the desired concentration, usually 0.1%, 0.2% or 0.25%. The pH should be 7.2-7.4.

Trypan Blue

Physiological saline (0.85%):

NaCl	0.85 g
Distilled water	100.00 ml

Trypan Blue (0.4%):

Trypan blue dye	0.4 g
Physiological saline	100.00 ml

Paper-filter before use. Concentrations of 0.1% and 0.2% are also used.

IUdR (5-Iodo-2-Deoxyuridine)

Dissolve IUdR in sterile distilled water to a final concentration of 1 mg/ml. This is a stock solution. For example: 0.1 g (IUdR) in 100 ml water.

Dispense in convenient amounts, 1 ml for example, and store at −20° C. Add the stock solution to the cell suspension to a final concentration of 10 μg/ml (1:100 dilution).

DEAE-D (Diethylaminoethyl-Dextran)

Dissolve DEAE-D in distilled water to a final concentration of 1 mg/ml, for example, 0.1 g in 100 ml water.

Autoclave 20 minutes. Cool and store at 4° C.

Dilute the stock solution 1:33 in Hanks' BSS to a final concentration of 30 μg/ml.

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Cycloheximide

Lots of cycloheximide vary in activity; therefore, each lot should be pretested and its optimal working concentration determined.

Prepare a stock solution by reconstituting the dry power with distilled water. The stock solution should be of a concentration that, when added to the medium, no more than 1 ml will be required to achieve the optimal working concentration in 100 ml of medium.

Sterilize the stock solution by membrane (0.2μm) filtration. Store in the dark at 4° C.

At the time of use, asceptically add the amount of stock solution to the medium that is required to obtain the predetermined optimal, working concentration (usually 1 or 2 μg/ml).

Example: Stock solution = 400 μg/ml
0.5 ml is added/100 ml medium
Final concentration - 2 μg/ml

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Giemsa Stain

Stock Solution:

Giemsa powder	0.5 g
Glycerine	33.0 ml
Absolute methanol	33.0 ml

Dissolve the powder in the glycerine by placing the mixture in a water bath (55°- 60° C) for 90 minutes. When crystals are dissolved, add 33 ml of absolute methanol. Store at room temperature.

Working Solution: Prepare fresh by diluting the stock 1:23 in phosphate buffer. Water, depending on its quality, may also be used for preparing the working solution.

Phosphate Buffer

Stock Solutions:

Na_2HPO_4	9.47 g
Distilled water q.s. to	1,000.0 ml
KH_2PO_4	9.08 g
Distilled water q.s. to	1,000.0 ml

Store at 4° C.

10X Buffer Concentrate:

Stock Na_2HPO_4	72.0 ml
Stock KH_2PO_4	28.0 ml

Store at 4° C.

Working Solution: Prepare fresh daily by diluting the 10X concentrate 1:10 in distilled water.

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Alcohol/Formalin Fixative

Formalin (37% formaldehyde)	100.0 ml
Absolute methanol (ethanol)	900.0 ml
Calcium acetate	0.5 g
(Calcium acetate · H_2O)	(0.57 g)

Combine reagents. Store at room temperature.

Jones' Iodine

Potassium iodide	5.0 g
Iodine crystals	5.0 g
Absolute methanol	50.0 ml
Distilled water	50.0 ml

Combine reagents and mix until dye is in solution. Store at room temperature in a brown bottle protected from light.

Before use, filter through a #41 ashless Whatman filter paper.

Iodine Mounting Medium

Equal parts Jones' iodine and glycerine

Glycerol Mounting Medium

Glycerol	9 parts
0.01 M PBS	1 part

Combine and adjust pH to 7.2.

Carbol Fuchsin

Stock Solution:

10% (W/V) Basic fuchsin in 95% ethanol	100 ml
4% (W/V) Aqueous phenol	250 ml
Distilled water	650 ml

0.1 M Sodium Phosphate Buffer, pH 7.45:

0.2 M NaH_2PO_4	3.5 ml
0.2 M Na_2HPO_4	15.5 ml
Distilled water	19.0 ml

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Working Solution:

Stock fuchsin	4.0 ml
Buffer, pH 7.45	10.0 ml

Filter immediately, and again just before use. The working solution remains satisfactory for 40 hours.

Malachite Green

Malachite green powder	0.8 g
Distilled water	100.0 ml

Dissolve the powder in water. Store at room temperature.

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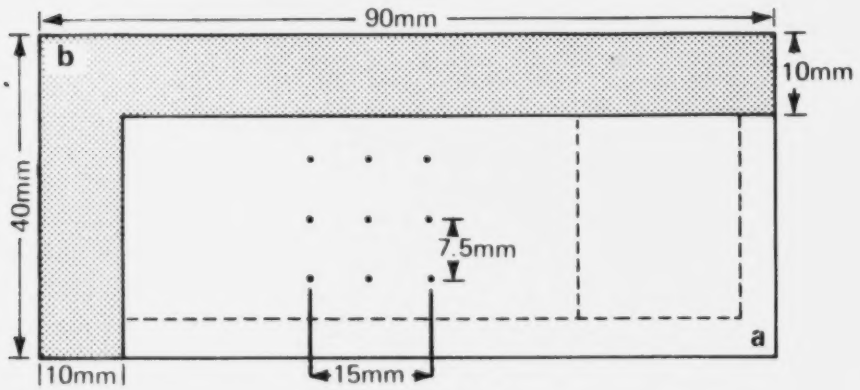
Preparation of Template

The template is made from 2 pieces (a and b) of construction board or similar material. The material used for piece (b) should be sufficiently thick to restrain a standard microscope slide.

1. Using the measurements in the illustration below as a guide, cut pieces (a) and (b).
2. Glue piece (b) onto piece (a) as illustrated.
3. With a pen, make 9 dots as in the pattern below. Each dot is 7.5 mm from adjacent dots. The 9 dots form a 15-mm x 15-mm square.



ACTUAL SIZE

Conjugates

Conjugates for the indirect IF test are available commercially. Before use in a diagnostic test, conjugates should be pretitered and the optimal working dilution determined.

Although the CDC manual *Immunofluorescence Methods in Virology* was developed for use in virology, it contains material that can be applied to Chlamydia IF. Subject matter of the manual includes sections on the fluorescence microscope, preparation of FITC-conjugates, and evaluation of conjugates.

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